The Regulation of Mucociliary Activity by Substance P Released from Airway Afferents

An Experimental Study in the Rabbit Maxillary Sinus

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Abstract

The physiological regulation of activity in the mucociliary (m.c.) system is not known in detail, although experiments with pharmacological compounds acting on autonomic receptors indicate regulation by the autonomic nervous system. M.c. activity is increased by short-term exposure to irritants and by inflammatory mediators. The mechanisms behind these responses are not known. The existence of various peptides in sensory fibres and in the autonomic nervous system suggests that some of them may modulate m.c. activity. Particularly effects of substance P (SP) in sensory C-fibres, which has been shown to mediate neurogenic inflammatory responses, deserve attention.

The m.c. activity in the rabbit maxillary sinus was recorded by a photoelectric method and the m.c. wave frequency analyzed by computer.

SP, or SP-like peptides were found to be present in subepithelial fibres in the rabbit maxillary sinus, in the maxillary nerve as well as in fibres in the sphenopalatine ganglion.

Exogenous SP accelerated m.c. activity. The effect was not mediated via muscarinic receptors nor influenced by the ganglionic blocker hexamethonium.

The effect of SP was inhibited by the SP antagonist (D-Pro^2, D-Trp^{7,9})SP. The blockade was reversible and did not affect muscarinic receptors.

Bradykinin and capsaicin, which are known to stimulate C-fibres in the airways and to release SP, mimicked the effect of SP on m.c. activity.

Antidromic nerve stimulation, known to stimulate C-fibres, increased m.c. activity. The effect of nerve stimulation was not influenced by previous cervical sympathectomy, but was abolished by pretreatment with (D-Pro^2, D-Trp^{7,9})SP.

Pretreatment with atropine suppressed the initial response to bradykinin, capsaicin and antidromic nerve stimulation, suggesting that C-fibres are the afferent component of a reflex arc with cholinergic effector neurons as the efferent pathway.

The increase in m.c. activity seen after short-term exposure to cigarette smoke was mediated by the above-mentioned reflex, since the response was suppressed not only by atropine, but also by (D-Pro^2, D-Trp^{7,9})SP and by pretreatment with capsaicin (in order to desensitize C-fibres). Furthermore, the non-cholinergic response to cigarette smoke was inhibited by (D-Pro^2, D-Trp^{7,9})SP. Taking into account the strong stimulating effect of SP on m.c. activity, this indicates that the m.c. response to irritants reflects the joint peripheral release of both SP and acetylcholine. This mechanism may be of major importance in the control of m.c. activity.

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University Hospital, Lund, Sweden

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The present thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

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II. Lindberg S, Mercke U.
Substance P Antagonists and Mucociliary Activity in Rabbit.
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III. Lindberg S, Mercke U.
Bradykinin Accelerates Mucociliary Activity in Rabbit Maxillary Sinus.
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IV. Lindberg S, Mercke U.
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V. Lindberg S, Mercke U.
Antidromic Nerve Stimulation Accelerates Mucociliary Activity in Rabbit Maxillary Sinus.
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VI. Lindberg S, Mercke U, Uddman R.
The Morphological Basis for the Effect of Substance P on Mucociliary Activity in Rabbit Maxillary Sinus.
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VII. Lindberg S.
Reflex-induced Acceleration of Mucociliary Activity after Short-term Exposure to Cigarette Smoke.
Submitted to Eur J Respir Dis 1985.

Abbreviations

ACh	Acetylcholine
CBF	Ciliary beat frequency
CGRP	Calcitonin gene-related peptide
GRP	Gastrin releasing peptide
i.a.	intraarterial
i.v.	intravenous
M.c. (or mc)	Mucociliary
NPY	Neuropeptide Y
PHI	Peptide with N-terminal histidine and C-terminal isoleucine
SD	Standard deviation
SEM	Standard error of mean
SK	Substance K (also called neurokinin A)
SP	Substance P
SPG	Sphenopalatine ganglion
TG	Trigeminal ganglion
VIP	Vasoactive intestinal polypeptide

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Introduction

The mucociliary (m.c.) system is one of the most important defence mechanisms in the respiratory tract maintaining stasis between the individual and the environment. Inhaled particles, bacteria and cellular debris are trapped in the mucus and transported by the constantly beating cilia to the pharynx where they are swallowed or expectorated. The wave movements produced by the beating cilia in the covering mucus are an expression of the m.c. activity of the mucous membrane. Factors influencing the structure and function of the cilia themselves, and/or the composition, and in turn the properties, of the respiratory secretions will produce changes in this activity.

Our knowledge of the physiological regulation of the m.c. activity is limited, but evidence has accumulated during recent decades indicating that the m.c. system is under the control of the autonomic nervous system. M.c. activity and transport are increased by cholinergic mechanisms that can be reproduced by pharmacological stimulation in man and other mammals (Camner et al 1974, Wanner et al 1975, Hybbinette & Mercke 1982b) and by electrical stimulation of cholinergic nerves in the frog (Slaughter & Aiello 1982, Morley & Sanjar 1984). It has also been reported that β_2-agonists like terbutaline and salbutamol increase m.c. activity and clearance (Camner et al 1976, Foster et al 1976, Hybbinette & Mercke 1982c). However, there are no reports of stimulation of sympathetic nerves so the physiological significance of the effects of β_2-agonists remains to be explained. The recent demonstration of neuropeptides in sensory nerve fibres and in the autonomic nervous system suggests the possibility that some of these peptides may influence the m.c. system.

Increased m.c. activity after exposure to irritants or during inflammatory conditions seems physiologically desirable. It has been reported recently that ciliary beat frequency is accelerated *in vitro* by inflammatory mediators, such as prostaglandins, leukotriene C_4 and histamine (Wanner et al 1983). Short-term exposure to a common irritant like cigarette smoke increases m.c. activity and clearance (Camner et al 1971b, Albert et al 1975, Hybbinette 1982). The mechanism by which inflammatory mediators and irritants stimulate m.c. activity is unknown.

The neuropeptide substance P (SP) mediates neurogenic inflammatory responses by release from peripheral sensory nerve endings (see Lembeck & Gamse 1982). Since SP stimulates glycoprotein production *in vitro* in the airways (Baker et al

1977), it is conceivable that SP and sensory nerve endings may also influence m.c. activity. SP is present in sensory nerve fibres in both the upper and lower airways (Lundberg et al 1983a, Lundblad et al 1983b, Uddman et al 1983).

It is of interest not only from a physiological but also from a clinical point of view to study the control of m.c. function. Further knowledge of the regulation of the m.c. activity may assist in the development of new treatments for various troublesome clinical conditions such as chronic bronchitis, cystic fibrosis and asthma in the lower airways, or chronic sinusitis and otitis media with effusion in the upper airways.

Morphological Background

A. COMPOSITION OF THE MUCOUS MEMBRANE IN THE AIRWAYS

The ultrastructure of the cilia is common to a variety of organisms throughout the whole animal kingdom and has survived millions of years of evolution. In man, cilia function in the brain ventricles and in the genito-urinary and respiratory tracts. A cilium consists of a ring of nine outer doublet microtubules, surrounding a central pair of single microtubules (Satir 1974). Each doublet consists of a subfibre A and a subfibre B, with dynein arms projecting from subfibre A towards the next microtubule's subfibre B. ATPase activity in the dynein protein provides the energy necessary for ciliary motion (Gibbons & Gibbons 1973). Cyclic detachment and attachment of the dynein arm to the microtubules results in a sliding movement which in turn bends the cilium (Satir et al 1981).

Ultrastructural abnormalities in the cilia (mainly missing dynein arms) are associated with male sterility (Pedersen & Rebbe 1975) and Kartagener's syndrome (the triad of bronchiectasis, chronic sinusitis and situs inversus) (Afzelius 1976, Pedersen & Mygind 1976). Similar structural changes are also found in groups of individuals with longstanding histories of bronchitis including bronhiectasis, chronic sinusitis and chronic otitis media with effusion (Eliasson et al 1977, Rossman et al 1980). Some of these may have a family history of situs inversus. This condition (which includes the Kartagener patients) is known as 'immotile cilia syndrome' or 'primary ciliary dyskinesia'. However, despite frequent airway infections and sterility (males) or reduced fertility (females), the syndrome seems to be compatible with normal lifespan.

Respiratory tract cilia are 5 to 7 μm long (Proctor 1982, Sanderson & Sleigh 1981b) and have a diameter of about 0.25 μm. Each ciliated cell has from about 50-100 cilia (in the nose) to 250 cilia (in the trachea) projecting from the luminal surface. They beat at a rate of about 1000 strokes per minute, and they propel materials at a speed of 3-25 mm/min (Proctor 1982). Both the beat frequency and the speed vary between individuals and within the same individual. In the upper respiratory tract ciliated mucous membrane lines the nasal cavity, paranasal sinuses, nasopharynx, auditory tubes and middle ear. In the lower airways it extends from the larynx to the ends of the terminal bronchioles.

The mucous membrane of the airways mainly consists of a pseudostratified ciliated epithelium resting on a basal membrane and an elastic lamina propria mucosae. The submucosa

is made up of loose connective tissue containing fat cells, blood vessels and submucosal glands (Jeffery & Reid 1977). Although the cilia are strictly uniform in different locations and species, the submucosal contents and various secretory cells are not. This means that one has to be very careful when generalizing results from observations of the m.c. system in the lower airways to the upper airways and vice versa and also between species.

This thesis is based on investigations in the rabbit maxillary sinus. The mucosa of the rabbit maxillary sinus consists of a pseudostratified (2 or 3 layers) ciliated epithelium with ciliated cells, mucous cells (also called goblet cells), basal cells and some intermediate cells on a basal membrane (Addicks et al 1982). The existence of goblet cells in the rabbit maxillary sinus has been questioned (Kumlien & Schiratzki 1985), in any case ciliated cells outnumber other types of cells in the sinus. Below the basal membrane is the lamina propria, which is close to the periost of the adjacent facial bones. The submucosa contains numerous glands with efferent ducts and vessels of different diameters. In contrast, the maxillary sinus of man has very few glands, most of them located around the ostium (Tos 1982).

The cilia on the ciliated cells beat with a fast effective stroke and a slower recovery stroke. The motion of adjacent cilia is coordinated (metachronism), ensuring a minimum of interference between adjacent cilia (Sanderson & Sleigh 1981a). In the airways the effective stroke is directed towards the pharynx, i.e. in the paranasal sinuses towards the ostium.

Cilia need a fluid medium. Already in 1934 Lucas & Douglas proposed that secretions in the airways, which is the medium in which cilia beat, consist of two layers. The cilia themselves are surrounded by an inner periciliary layer of more serous fluid, and an overlying viscoelastic mucus layer, produced by submucosal glands and secretory cells (mucous and serous cells) in the epithelium (Reid et al 1982) (Fig. 1). During the effective stroke the cilia are extended so that their tips, which appear to be 'clawed' (Dirksen & Satir 1972), reach the overlying mucus and move it in the direction of the effective stroke.

The secretions forming the periciliary layer probably originate from the ciliated cells themselves (Reid et al 1982). In this context, it is interesting that both ciliated and non-ciliated epithelial cells in the airways are provided with microvilli and share characteristics with other secreting cells elsewhere in the body (Rhodin 1966, Petruson et al 1984). The periciliary fluid is probably the result of active ion transport by epithelial cells (Olver et al 1975), secondarily increasing the water content as a result of the osmotic gradient produced. An electrical potential difference has been demonstrated across the airway mucosa both *in vitro* and *in vivo*, in several species including man (Nadel & Davis 1978, Knowles et al 1981). The lumen is always negative to the mucosa.

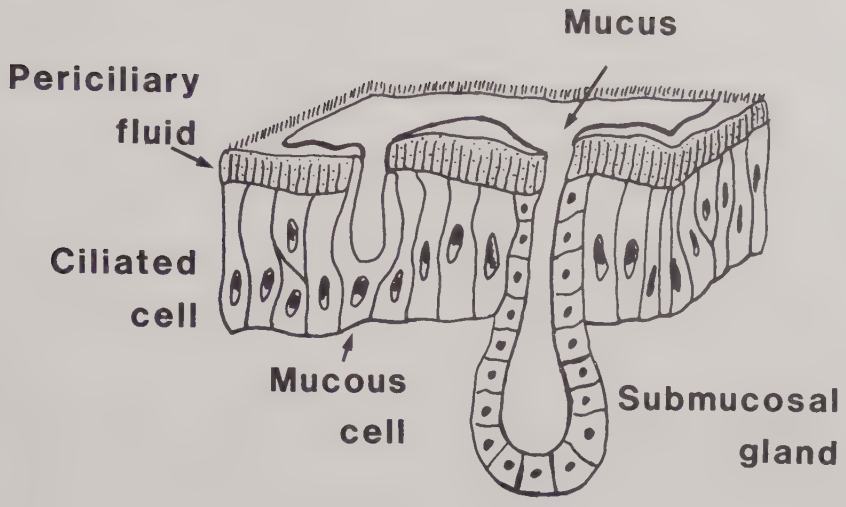

Fig. 1. Schematic illustration of the cilia beating in the periciliary fluid, which probably derives from the ciliated epithelium itself. The work of the beating cilia transports the overlying viscoelastic mucus consisting of secretions mainly from submucosal glands and secretory cells in the epithelium (mucous and serous cells).

This potential difference results from a net movement of Cl^- towards the lumen, and a smaller net movement of Na^+ towards the submucosa. There is probably a linked entry of Na^+ and Cl^- across the basolateral membrane of the epithelial cell. Na^+-K^+ATPases actively pump Na^+ from the cell, while Cl^- diffuses down its electrochemical gradient across the luminal membrane. Any stimulus that increases net movement of Cl^-, but not of Na^+, towards the lumen will tend to increase the water content of the respiratory secretions and in turn affect m.c. function (Nadel & Davis 1978, Widdicombe & Welsh 1980).

B. INNERVATION

According to Cauna et al (1969), the sensory innervation of the nasal mucosa in man is mediated by non-myelinated fibres exhibiting acetylcholinesterase activity. They terminate in a simple arborization in the lamina propria that partly extends into the epithelium. They originate from the ophthalmic (fronto-superior part of the nose) and maxillary (posterior-inferior part) divisions of the trigeminal nerve (Cauna 1982). There are similar axon bundles in the rabbit maxillary sinus, mainly unmyelinated with terminal varicosities lying close to the respiratory ciliated epithelium and the glandular epithelium (Addicks et al 1982). Whether these axons represent sensory fibres or autonomic effector neurons is not known. The numerous small electron-lucent vesicles and some large granular vesicles found in the endings occur in both types of neurons.

The transmitter in some of these unmyelinated nerve endings (considered to be C-fibres with slow propagation of impulses of 0.8-2.4 m/s, Coleridge & Coleridge 1977) may be substance P (SP), as proposed by Hökfelt et al (1975). SP belongs to a group of neuropeptides called tachykinins. Other members of this group are neurokinin A (also called substance K) and neurokinin B (Kangawa et al 1983, Nawa et al 1984). The number of SP fibres in the nasal mucosa diminishes after treatment with high doses of capsaicin (Lundblad et al 1983b), the active principle in paprika, *Capsicum annuum* and chili peppers, *Capsicum frutescens*. It is known that capsaicin depletes SP from peripheral sensory nerve endings (Gamse et al 1980), and causes degeneration of unmyelinated sensory neurons (Jancsó et al 1977). SP immunoreactive nerve fibres have been demonstrated in the nasal mucosa of many species, where fibres are found around blood vessels and seromucous glands in the submucosa and in the subepithelial layer, sometimes penetrating the epithelium. The majority of the SP fibres originate in the trigeminal ganglion, where SP-positive nerve cell bodies are found (Lundblad et al 1983b, Uddman et al 1983). SP-immunoreactive nerve fibres and occasional nerve cell bodies are also found in the sphenopalatine ganglion (Uddman et al 1983). The presence of SP in paranasal sinuses has not been demonstrated.

Unmyelinated C-fibres in the airways may also contain substance K (SK), which is released from guinea-pig lung tissue after challenge with capsaicin (Saria et al 1985). The co-existence of SP and a third peptide, calcitonin gene-related peptide (CGRP) has been recently demonstrated (Lundberg et al 1985).

Efferent autonomic impulses to the mucous membrane in the nose and the paranasal sinuses are transmitted by parasympathetic and sympathetic effector neurons. The parasympathetic post-ganglionic nerve fibres to the upper respiratory tract are derived from the sphenopalatine ganglion, which in the rabbit

is a thin leaf-like structure adjacent to the medial bony wall of the orbit (Ruskell 1965). The preganglionic supply emerges from the superior salivary nucleus via the major petrosal nerve which is a branch of nervus intermedius, a division of the facial nerve. The postganglionic fibres innervate submucosal glands and blood vessels in the nasal mucosa, paranasal sinuses, pharynx and Eustachian tube. The transmitter in parasympathetic neurons is acetylcholine, acting at the preganglionic level on nicotinic receptors and at the postganglionic level on muscarinic receptors. However, the postganglionic neurons contain also vasoactive intestinal polypeptide (VIP) (Lundberg et al 1980), and another peptide PHI as well (the peptide with N-terminal histidine and C-terminal isoleucine), structurally related to VIP. In fact, most neurons in the sphenopalatine ganglion display both VIP and PHI immunoreactivity (Lundberg et al 1984).

Sympathetic postganglionic nerve fibres emerge from the superior cervical ganglion, whereas the preganglionic nerve fibres originate in the lateral horns of the grey medulla of the spinal cord. These fibres innervate mainly the vasculature via paravascular plexuses (Dahlström & Fuxe 1965, Cauna et al 1972, Ånggård & Densert 1974) and few adrenergic fibres occur near nasal submucosal glands. Instead the secretory activity of the glands may be under sympathetic influence through their blood supply. The transmitter in postganglionic sympathetic nerves is noradrenaline, whereas the preganglionic transmitter is acetylcholine acting on nicotinic receptors. A population of the sympathetic nerve fibres innervating the upper airways and emerging from the superior cervical ganglion, contains neuropeptide Y (NPY) (Ekblad et al 1984). These neurons mainly innervate arteries and arterioles, but only exceptionally veins.

In the rabbit maxillary sinus, catecholamine fluorescence is found in nerve fibres around blood vessels in the lamina propria, while no adrenergic nerves are found close to the epithelium. Acetylcholinesterase-positive nerve fibres, suggesting parasympathetic (cholinergic) innervation, can be demonstrated around secretory end-pieces, efferent ducts, blood vessels and sometimes in the ciliated epithelium (Schindelmeiser et al 1982). However, it has been questioned whether acetylcholinesterase-staining truly represents neurons with acetylcholine as the transmitter (Silver 1974), since cholinesterases are widely distributed enzymes. It is known that sensory neurons contain acetylcholinesterase (Cauna et al 1969, Tervo et al 1983) and some of the acetylcholinesterase neurons described by Schindelmeiser et al may therefore be sensory rather than parasympathetic.

Control of Mucociliary Function

A. PHARMACOLOGICAL INFLUENCES

A vast number of compounds have been shown to have in vivo and in vitro effects on m.c. function such as general and local anesthetics, opiates, ethyl alcohol, methylxanthines, cardiac glycosides, antimicrobial drugs and mucolytic agents (see Wanner 1977). Although some of these effects (especially the depressant ones of general and local anesthetics) have clinical importance, the effect of these compounds seems to be more pharmacological than physiological. In the following, only drugs whose effects on the m.c. system reflect a putative physiological role in the regulation of m.c. function will be described.

M.c. activity and transport can be influenced by action on the mucus secretory apparatus, which in turn changes the rheological properties of the mucus and periciliary layer, or by direct action on the cilia. It is difficult to determine which effect dominates in a given situation, because factors that affect the cilia directly may simultaneously influence the composition of the periciliary layer.

Parasympathomimetic drugs, acting on muscarinic receptors, generally stimulate m.c. activity and transport regardless of species and methods of measurement (Kordik et al 1952, Albert et al 1973, Camner et al 1974, Iravani & Melville 1975a, Wanner et al 1975). Isolated cell preparations suggest a direct effect on the ciliated epithelium itself (Corssen & Allen 1959). Reports of the effect of the muscarinic antagonists atropine and scopolamine on basal m.c. function are contradictory. Unchanged m.c. clearance has been demonstrated (Camner et al 1974), but also decreased activity and transport (Corssen & Allen 1959, Blair & Woods 1969, Annis et al 1976, Giordano et al 1978). Local application of ipratropium bromide (Atrovent) does not seem to change m.c. clearance in therapeutic doses (Pavia et al 1979). In the present experiment model the parasympathetic drug methacholine increases m.c. activity (maximum increase being about 50%), whereas atropine has no effect on the basal m.c. wave frequency (Hybbinette & Mercke 1982b).

The effect of sympathomimetic compounds on m.c. function is more complex than that of drugs acting on muscarinic receptors. It is generally agreed that β-agonists increase m.c. transport both under normal conditions and under conditions with impaired m.c. activity (Santa Cruz et al 1974, Camner et al 1976, Foster et al 1976, Sackner et al 1976). This effect is seen after treatment with unselective

β-agonists (like isoprenaline) or with any β_2-agonist (like terbutaline or salbutamol). The effect is probably mediated through β_2-adrenoceptors since prenalterol, which mainly acts on β_1-adrenoceptors does not influence m.c. activity (Hybbinette & Mercke 1982c). On the other hand, noradrenaline does not influence m.c. function (Hybbinette & Mercke 1982a, Morley & Sanjar 1984) suggesting that increased m.c. activity and transport seen after β_2-stimulation are a pharmacological rather than physiological effect. Despite that, it must be emphasized that β_2-agonists are the only drugs that for the moment have a well-documented stimulating effect on the m.c. system and that are prescribed for respiratory diseases.

In the present experiment model, isoprenaline and salbutamol increase m.c. activity. However, this is less prominent than the effect of methacholine (maximum frequency increase for salbutamol is about 25%). The response to the β_2-agonists has a latency of 30-40 sec versus 6-10 sec for methacholine. The unselective β-adrenoceptor antagonist propranolol does not influence m.c. wave frequency (Hybbinette & Mercke 1982c).

The effect of α-agonists on the m.c. system has not been thoroughly studied, although the compounds are very common in various decongestants and nose drops. Some investigations have demonstrated retarded m.c. transport (Simon et al 1977, Peerless & Noiman 1980), but unchanged (Petruson & Hansson 1982) and even increased m.c. transport have been reported (Saketkhoo et al 1978). In the rabbit maxillary sinus both α_1 and α_2-agonists decrease m.c. wave frequency, suggesting impaired m.c. function (Hybbinette & Mercke 1982c). This effect has been attributed to a decrease in the blood supply to the epithelium (Hybbinette & Mercke 1982c). Another possible explanation is a decrease in cyclic AMP produced by α_2-agonists (Exton 1981). Compounds that increase cyclic AMP are known to stimulate m.c. activity, for example salbutamol (Hybbinette & Mercke 1982c).

The bioactive amine serotonin stimulates ciliary beating frequency in the lamellibranch gill at low concentrations, and it has been suggested that serotonin is the endogenous pacemaker substance in molluscs (Gosselin et al 1962, Schor 1965). The effect of serotonin on the m.c. system in mammals does not seem to be important, although an *in vivo* stimulatory effect has been reported in cats (Dadaian et al 1971) and *in vitro* in the rabbit trachea (Tsuchiya & Kensler 1959).

Histamine plays a central role in anaphylactic reactions in the airways. Increased tracheal mucus transport has been reported in dogs challenged with nebulized histamine (Wanner et al 1975). Furthermore, histamine has a modest *in vitro* stimulatory effect on ciliary beating (Wanner et al 1983), and it also increases the transport of ions and water in the tracheal epithelium (Marin et al 1977). Bronchial challenge with ragweed extract to asymptomatic nonsmokers with ragweed asthma produced a decrease in tracheal mucus velocity (Ahmed et al 1981), which was reversed to an increase after pretreat-

ment with a slow reacting substance of anaphylaxis (SRS-A) antagonist, suggesting that SRS-A released during asthmatic attacks impairs m.c. function while other mediators, e.g. histamine, stimulate m.c. activity and transport.

SRS-A consists of the three leukotrienes LTC_4, LTD_4 and LTE_4. They are metabolites of arachidonic acid generated from phospholipids in cell membranes by the lipoxygenase way (see Bisgaard 1984). The assumption of Ahmed et al (1981) that leukotrienes impair m.c. function (vide supra) seems reasonable, but in vitro studies are conflicting. Both LTC_4 and LTD_4 increase mucus secretion from human airways in vitro (Marom et al 1982) and an effect of leukotrienes on m.c. activity might be expected. Bisgaard & Pedersen (1983) reported decreased activity in human ciliated cells challenged with LTC_4, whereas LTD_4 had no effect. In contrast to this, Wanner et al (1983) observed increased ciliary beating in sheep tracheal explants after exposure to LTC_4.

The products of the cyclooxygenase pathway of arachidonic acid metabolism, i.e. various prostaglandins, seem to have a ciliostimulatory effect. Prostaglandin E_1 increases mucus transport in rat trachea (Iravani & Melville 1975b) and prostaglandins E_1 and E_2 both increase ciliary beat frequency in sheep tracheal explants (Wanner et al 1983). Prostaglandin $F_{2\alpha}$ is without effect on the ciliary beating in the latter preparation. This prostaglandin increases mucus glycoprotein release from human lung explants, whereas prostaglandin E_2 interestingly reduces the release (Marom et al 1981). The discrepant effects on mucus production and m.c. activity of arachidonic acid metabolites deserve further investigations.

The basic peptide bradykinin is generally regarded as an inflammatory mediator. A kallikrein-like substance has been demonstrated in cat nasal secretion (Eccles & Wilson 1973a). Kallikrein is an enzyme that converts kininogen to bradykinin. The effect of bradykinin on m.c. activity is unknown, and reported secretory effects in airway mucosa are contradictory. There is no in vitro effect of bradykinin on canine mucus production (Baker et al 1977), but in vivo atropine-sensitive secretion from dog tracheal glands is stimulated by bradykinin (Davis et al 1982).

Another basic peptide involved in inflammatory reactions is substance P (SP), which in contrast to bradykinin produces glycoprotein secretion in canine tracheal explants (Baker et al 1977). SP occurs in some unmyelinated sensory C-fibres and has been put forward as a mediator of neurogenic inflammation in general (Lembeck & Holzer 1979). SP is released from the central endings of these sensory neurons upon stimulation (see Otsuka et al 1982) and also from the peripheral endings of sensory neurons. The physiological function of SP released from the central endings is probably to mediate pain (nociceptive function, Henry et al 1980). Stimulation of capsaicin-sensitive C-fibres activates various protective reflexes, such as the tail-withdrawal reflex to thermal

stimuli in mice (Gamse 1982) or the sneeze reflex in guinea--pigs (Lundblad et al 1984).

The terminal fibres of SP neurons are excited by noxious stimuli (in particular chemical or thermal). Any lesion in the airway mucosa can be expected to excite these neurons. The response travels orthodromically to the brain stem or spinal cord, and antidromically in the peripheral branches of the stimulated neuron, where SP is released (see Lembeck & Gamse 1982). The older concept of this axon reflex arrangement with separate afferent and efferent branches in the same neuron, has thus been replaced by the interpretation that the nerve endings themselves have a dual sensory-efferent function (see Szolcsányi 1984).

Exogenous SP and C-fibre stimulation is known to cause vasodilation and plasma extravasation in the upper airways (Lundblad et al 1983a,c) and oedema and bronchoconstriction in the lower airways (Lundberg & Saria 1983, Lundberg et al 1983b). Some of these effects are classical parts of the inflammatory response, i.e. rubor, calor and tumor. Interactions in mucosal defence between inflammatory mediators and this neurogenic mechanism are possible, since bronchial C-fibres are stimulated by inflammatory mediators, such as prostaglandins, histamine and bradykinin (Coleridge et al 1978, Kaufman et al 1980). An effect of SP on m.c. activity has hitherto not been reported.

B. NERVE STIMULATION

Investigations regarding the effect of nerve stimulation on m.c. activity and transport are few, probably owing to technical difficulties. After the study of Lucas & Douglas (1935) in the turtle, where nerve stimulation (and pharmacological challenges) was without effect on m.c. activity, it was believed for a long time that the ciliated cells of the respiratory tract of vertebrates, with the exception of the frog, were generally unresponsive to nerve stimulation (Gosselin 1966).

Offering a relatively simple and stable in vivo model, the frog palate has been widely used for investigations of m.c. function. Already in 1931, Seo demonstrated a reflex arc with the glossopharyngeal nerve beneath the tongue as the afferent pathway and the palatine nerve as the efferent pathway. When the glossopharyngeal nerve endings were stimulated, m.c. transport increased on the palate. Slaughter & Aiello (1982) investigated this reflex further and included experiments with direct stimulation of the effector nerve. M.c. transport, ciliary beating and mucus secretion from goblet cells increased after nerve stimulation with impulses of 1.5 V, 10 Hz, 0.8 ms. These effects were markedly reduced by atropine, indicating that the effect was mediated by acetyl-

choline on muscarinic receptors. Morley & Sanjar (1984) reported a similar finding in the frog oesophagus, where electrical stimulation (impulses of 0.5-1.5 V, 30 Hz, 2 ms) increased the rate of particle transport. This effect of nerve stimulation was suppressed by atropine and hexamethonium, whereas catecholamines were without effect on the increased particle transport rate due to stimulation of the vagus. The effect of electrical nerve stimulation on m.c. activity in mammals is unknown. However, indirect evidence from experiments regarding mucus secretion in the upper airways suggest that nerve stimulation may affect m.c. activity in mammals. Stimulation of the Vidian nerve in cat promotes secretion which is blocked by atropine, indicating that the response is mediated by cholinergic fibres (Eccles & Wilson 1973b, Änggård 1974). Also sympathetic electrical nerve stimulation causes secretion in the cat (Wilson & Yates 1978) and in the rabbit (Pell et al 1984).

C. IRRITANTS

Harmful effects of irritants on m.c. function in the respiratory tract epithelium were recognized several decades ago. Among irritants that impair m.c. activity and transport are cigarette smoke, ammonia, sulphur dioxide and formaldehyde (Mendenhall & Shreeve 1937, Dalhamn 1956, Hilding 1956). The effects of the irritants include ciliostasis (Dalhamn 1956) and modified glycoprotein composition (Jones & Reid 1978). Prolonged exposure to irritants leads to morphological changes including secretory cell hyperplasia (Reid 1954, Jones & Reid 1978), loss of ciliated cells and squamous metaplasia (Auerbach et al 1967). Many of the investigations on the m.c. effect of irritants were performed *in vitro* or in the opened trachea of experimental animals, thereby by-passing the upper airway and the mouth where irritants can be absorbed (Dalhamn et al 1968). *In vitro* preparations have the drawback that the mucosa is deprived of both circulation and innervation. Furthermore, irritants are known to activate protective cardio-respiratory reflexes that originate in the nose (Angell James & de Burgh Daly 1969, McRitchie et al 1970). These reflexes may in turn influence m.c. function.

Consequently, the *in vivo* effects of irritants on the m.c. system are more complicated than the previous investigations indicated. In a recent review, Lippmann & Schlesinger (1984) conclude that the response of the m.c. system to short-term exposure to several irritants seems to be accelerated mucus flow rate at low concentrations, and a retardation at higher levels (or prolonged exposure). These irritants include cigarette smoke (Camner et al 1971b, Albert et al 1975), sulphur dioxide (Spiegelman et al 1968, Wolff et al 1975) and sulphuric acid (Wolff et al 1981, Lippmann et al 1982, Chen & Schlesinger 1983). Short-term exposure to cigarette smoke accelerates m.c. activity in rabbit maxillary sinus

(Hybbinette 1982). The similarities between the effects of various irritants suggest a nonspecific response (Lippmann & Schlesinger 1984).

Cigarette smoke and ammonia increase mucus secretion measured with radioactive labels, (^{35}S) sulphate and (^{3}H) glucose, in cat trachea and rabbit nose (Richardson et al 1978, Pell et al 1984). The effect in cat trachea seems to be mediated by two pathways (Phipps 1980):

1. A reflex, neural pathway, probably involving the stimulation of tracheo-bronchial cough receptors.

2. A direct action on the mucus-secreting cells, possibly involving local release of a chemical mediator. Combined blockade with atropine and propranolol only partly inhibits the response (Richardson et al 1978).

Autoradiographic studies demonstrate that ammonia increases secretion from the epithelial lining (presumably the surface mucosubstance according to Spicer et al 1971) rather than from submucosal glands (Jeffery 1978). Moreover, the parasympathomimetic drug pilocarpine exhausts submucosal glands while the surface mucosubstance remains intact (Jeffery 1978). This suggests that secretion from submucosal glands is mainly under cholinergic control, whereas secretion from the epithelial lining is stimulated by irritants.

In Vivo Methods for Measuring Mucociliary Function

A. RECORDING TECHNIQUES

The methods for measuring m.c. function may be subdivided according to whether one records m.c. activity (or more precisely the frequency of the waves brought about by the beating cilia, the m.c. wave frequency) or the m.c. transport and clearance rates that result from the beating.

Measurement of m.c. wave frequency requires an indirect light technique. A light beam (usually from a cold-light source) directed at the epithelium at an angle to the visual axis, is reflected by the cilia and by the mucus covering the cilia (Toremalm et al 1974). The frequency of the flickering reflection can be measured by several methods, e.g. stroboscopy (Gray 1931), high-speed photography (Dalhamn 1956, Sanderson & Sleigh 1981a) and with the aid of a photo-electric cell (Dalhamn & Rylander 1962, Guillerm et al 1965, Håkansson & Toremalm 1965, Mercke et al 1974). These methods have in common the advantage that markers are not necessary and thus mechanical disturbance introduced by the recording technique itself is minimized. However, the usefulness of the three methods differs. Stroboscopic methods do not register rapid frequency changes. Cinematographic methods, although very useful for detailed studies of metachronism (Sanderson & Sleigh 1981a), are time-consuming and expensive, in particular during in vivo studies when m.c. activity is observed for several hours. The photo-electric technique is simpler, and permits immediate detection of frequency changes and continuous recording for several hours. Furthermore, the electrical signals are suitable for computerized analysis. The main limitation of the method is that it gives little information about the efficiency of the m.c. system (Phipps 1981).

Other methods used in m.c. investigations measure the result of the work of the beating cilia, i.e. the transportation of various materials. Two different methods are used; m.c. transport of markers placed on the mucosa and m.c. clearance of inhaled particles.

M.c. transport can be measured as the time needed for a marker placed on the mucus to be transported a given distance, or as the distance a marker is moved during a fixed period of time. Marker substances include coloured solutions and dyes, saccharine, lycopodium, carbon particles, cellular debris, teflon discs and particles labelled with radioisotopes (e.g. ^{99}Tc) (see Wanner 1977, Proctor 1982). The transport technique

has been widely used for in vivo studies (Dalhamn 1956, Ewert 1965, Simon et al 1977, Slaughter & Aiello 1982, Morley & Sanjar 1984).

Phipps (1981) has pointed out some problems of this method. Many marker particles are large (often 0.5-1.0 mm in diameter) and not representative of natural contaminants. If only one or two particles are monitored their placement is critical. Slow zones and fast zones have been observed, so a small difference in particle placement from one trial to another may greatly influence the results. Furthermore, the radioactive decay of radioactively labelled particles may itself alter mucus transport and m.c. activity (Ahmed et al 1979, Baldetorp 1985).

M.c. clearance measures overall rate of removal of a known amount of deposited or inhaled tracers, radioactively labelled with a γ-emitting isotope. It has been used for studies of m.c. function in man (Albert et al 1967, Camner et al 1971a). The radioactivity is measured at regular intervals, and the results are expressed as percentages of the initial burden of radioactivity retained or cleared as a function of time. The small tracers (usually 1-10 µm in diameter) used in this method might penetrate into the mucus or even the periciliary layer. The clearance rate probably represents two different mechanisms, a fast phase virtually complete within 24 hours representing m.c. clearance and a slow phase (which takes months to complete) representing nonmucociliary mechanisms, possibly from alveoli (Phipps 1981).

One main problem of m.c. clearance studies is the deposition of the tracers, since clearance is faster in the central than in the peripheral parts of the respiratory tract (Morrow et al 1967). Cigarette smoking and obstructive lung disease cause a more central distribution of the inhaled tracer, giving the false impression of normal clearance in these subjects. The results in subjects with increased amount of tracheobronchial secretion are also influenced by coughing, which produces a stepwise decrease in the number of radioactive particles (Camner et al 1979). Moreover, the safety of radioisotopic techniques when studying the m.c. system in man can be questioned (Ahmed et al 1979).

Very few direct comparisons have been made between measurements of m.c. wave frequency (by photoelectric methods) and m.c. transport or clearance. However, Hée & Guillerm (1977) have reported a direct relation between ciliary beat frequency and m.c. transport rate in sheep trachea in vitro. Similarly Slaughter & Aiello (1982) observed that flickering and m.c. transport were both accelerated in the frog palate in vivo by cholinergic nerve stimulation. A change in the m.c. wave frequency does not necessarily reflect a change in mucus flow rate. However, an investigation in the present model has shown increased m.c. wave frequency after injection of the parasympathomimetic drug methacholine (Hybbinette & Mercke 1982b), and others have found increased m.c. transport or clearance after challenge with similar compounds (Albert et al 1973,

Camner et al 1974, Slaughter & Aiello 1982). Again, similar increases of m.c. wave frequency (Hybbinette & Mercke 1982c) and clearance (Camner et al 1976, Foster et al 1976) have been reported for β_2-stimulating compounds. Short-term exposure to cigarette smoke increases m.c. wave frequency in the rabbit maxillary sinus (Hybbinette 1982) as well as m.c. clearance in man (Camner et al 1971b, Albert et al 1975). Nevertheless, further research regarding this question is needed before the question of a correspondence between m.c. wave frequency and m.c. transport or clearance can be settled.

B. ADMINISTRATION TECHNIQUES

Test substances can be administered to the respiratory mucous membrane in vivo either locally or parenterally. The former has the advantage that it minimizes the risk of general side effects, which could cause unspecific influences on the m.c. system. Moreover, this procedure mimics the ones used for many drugs in clinical practice (nose drops, aerosol sprays etc). However, as locally applied test substances may change the rheological properties of the mucus, and as they may also influence m.c. function by tactile stimulation, this technique is less suitable for investigations of the physiological control of m.c. activity. Instead, the parenteral route is to be preferred for such studies, because it permits the test substances to be administered in reproducible doses to the effector cells without physical or chemical interference to other components of the m.c. system.

Aims of the Study

The physiological regulation of m.c. activity is incompletely understood, although experiments with pharmacological compounds acting on receptors belonging to the autonomic nervous system indicate a regulating role for autonomic nerves. The existence of various peptides in sensory nerve fibres and in the autonomic nervous system suggests that some of them may modulate m.c. activity. However, reports regarding the effects of neuropeptides on the m.c. system are lacking.

Previous investigations have demonstrated the existence of a nonspecific m.c. response to irritants, which probably involves a non-cholinergic and non-adrenergic mechanism (see pages 18-19). It is conceivable that this response is mediated by neuropeptides, especially SP and other tachykinins. Previous studies have indicated that neurons containing SP mediate protective mechanisms in the airways, and that they are involved in nervous responses to irritants and in neurogenic inflammation in general. The following aspects of the effects of SP and the role of SP-containing neurons in the control of m.c. activity was investigated in the rabbit maxillary sinus:

1. The effects on m.c. activity of four neuropeptides, whose presence in the upper airways has previously been demonstrated by immunocytochemical methods (I).

2. The suppression by SP antagonists of the effect of SP on m.c. activity and the selection from three available SP antagonists of one which has a distinct blocking effect (II).

3. The effects on m.c. activity of pharmacological stimulation with bradykinin or capsaicin of neurons believed to contain SP, i.e. unmyelinated C-fibre afferents (III, IV).

4. The effects of antidromic trigeminal nerve stimulation - known to release SP-like immunoreactivity from C-fibres - on m.c. activity (V).

5. The presence of SP-immunoreactive nerve fibres in the maxillary sinus, maxillary nerve and sphenopalatine ganglion of the rabbit (VI).

6. The role of capsaicin-sensitive C-fibres, and for SP released from them, in the m.c. response to a common airway irritant - cigarette smoke (VII).

Methods

Animals
White rabbits of either sex (Swedish Domestic or New Zealand White) wheighing 1.8-3.6 kg and raised under controlled conditions were used. Only healthy animals were accepted for the study.

Anesthesia
The animals were anesthetized by injection of urethane (0.5 g/ml) 2 g/kg i.m. as an initial dose and an extra dose of 0.5 g/kg was given in an ear vein during the operation. The urethane was sometimes supplemented by local anesthesia (1-2 ml) around skin incisions. Lidocaine hydrochloride 10 mg/ml (Xylocain, Astra, Sweden) or mepivacain hydrochloride 10 mg/ml (Carbocain adrenalin, Astra, Sweden) were used for this purpose. No further anesthesia was usually needed for 5-6 h. The criterion for adequate anesthesia was regular spontaneous breathing without spontaneous movements. Cervical sympathectomy (VII) was performed in a few rabbits anesthetized with mebumal 30-40 mg/kg i.v.

Preparation of the model
The facial or lingual artery was cannulated (Portex intravenous cannula, outer diameter 0.75 mm), the catheter being advanced in the vessel until the tip reached only a few mm from the orifice of the maxillary artery, which supplies the maxillary sinus. This means that the i.a. injections reached not only the maxillary artery but also other tributaries of the external carotid. The cannula was continuously perfused with physiological saline 2-5 ml/h (usually 2 ml/h) in order to keep the inlet open. This i.a. route was used for administering drugs.

The maxillary sinus was opened on the same side as the catheterization by drilling a trepanation hole of about 2x8 mm and an incision was made through the mucous membrane. The incised edges of the membrane were retracted to the margins of the trepanation. Extreme care was undertaken in order to avoid bleeding into the sinus during these moments, since any contamination of the mucosa with blood would clearly impair the following experiments. The opening was immediately covered with an anti-mist window through which the ciliated membrane could be studied. The window was sealed with bone-wax (Ethicon) in order to prevent dehydration. Sinus ventilation was thus restored to normal, i.e. only via the nasal cavity. To avoid condensation the window was heated slightly above body temperature by electrical resistances glued to the glass. The rabbit's head was stabilized in a specially built holder whose position could be adjusted by means of micrometer screws.

ECG was recorded simultaneously with the m.c. activity, and in some experiments with cigarette smoke (VII) the respiratory frequency was recorded by a HP Toccotransducer 15278 B. Rectal temperature was monitored (Ellab du3s) and body temperature was maintained within 37.0-38.5°C with the aid of a heating pad.

Recordings of mucociliary activity

Light from a cold light source (Heim L151) was directed obliquely to the mucosa in the fronto-superior part of the maxillary sinus so that it was reflected from the mucus blanket. The m.c. activity produced a flickering reflection, which was observed through an operating microscope (Zeiss, OPMI1). The criterion for a working preparation was visible transportation of small particles like mucus clumps and shed cells. One of the eyepieces was then replaced by a photo-transducer which transformed the light intensity variations into electrical signals. These were bandpass filtered at 3-30 Hz (Krohn-Hite 3550) and monitored continuously on an oscilloscope (Gould Advance OS 260). The responses to the challenges were recorded on an ink writer (Siemens-Elema Mingograph 803). A computerized frequency calculator analyzed the m.c. wave frequency every 10 sec during challenges and at intervals of 1-5 min otherwise. The frequency was expressed in waves/min. 10 sec readings were based on the frequency during the previous 10 sec, whereas 1 and 5 min readings were based on the frequency during the previous 20 sec. Occasional complementary manual analysis of the ink writer recordings was done as necessary.

The accuracy of the automatic frequency calculations by the computer was determined in 5 rabbits where 138 recordings of m.c. wave frequency were analyzed both manually and by the computer. These rabbits were challenged with various drugs (methacholine, SP, VIP, bradykinin, capsaicin, physiological saline) in order to obtain recordings representing most m.c. wave frequencies observed in the present thesis, i.e. from 900-2200 waves/min. The result of this comparison is shown in Fig. 2.

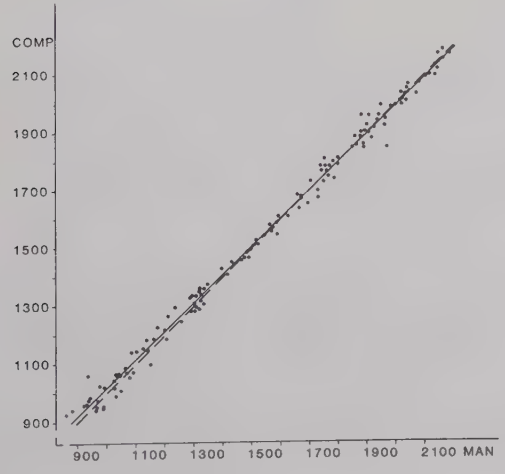

Fig. 2. The correlation between manual (MAN) and computer (COMP) analyses of 138 recordings of m.c. wave frequency. Frequencies are expressed in waves/min. The solid line (———) represents the actual linear regression and the broken line (– – –) the ideal relationship. The slope is 0.98, the correlation coefficient r=0.97 and F-test for regression $p<0.001$.

The agreement between manual and computerized calculations of m.c. frequency confirms the close correspondence between the two methods and justifies the use of computer technology in studies of m.c. activity.

Administration of drugs

In general drugs were dissolved in physiological saline, except the neuropeptides (including bradykinin) which were dissolved in saline containing 0.5% albumin (Kabi, Stockholm, Sweden) in order to prevent adsorption to glass and plastic surfaces. Polystyrene and polyacetate plastic were avoided for the same reason (Stewart 1983). Capsaicin (II, IV, VII) was dissolved in saline containing 10% Tween 80 and 10% ethanol, further dilutions were made in saline. Indomethacin was dissolved in sterile water.

The drugs were generally injected as 3 sec bolus doses of 0.2 ml in the arterial inlet. A somewhat larger bolus dose was used for guanethidine (IV), 0.5 ml in 10 sec. Arterial infusions, used for SP antagonists, were given in the same cannula for 4 min. The rate of infusion was 0.5-0.9 ml/min, depending on the size of the animal.

All i.v. infusions were given in an ear vein, while i.m. injections were performed in the gluteal region or the back of the animals. No drugs were applied locally.

Nerve stimulation (V)

The maxillary nerve was exposed by a modified version of the technique described by Änggård (1974). The eyeball was opened, emptied and the remainder retracted upwards and stay-sutured. The bottom of the orbit was then carefully dissected through an incision in the lower eyelid. The maxillary nerve, which courses medially to the alveolar tube, was isolated and cut high in the orbit and the distal end was laid across a bipolar platinum electrode. Possible connections between the sphenopalatine ganglion and the maxillary nerve might be damaged during this manoeuvre. The existence of such connections in the rabbit is unclear (Ruskell 1965). The cut nerve was covered with Plastibase (Squibb) or cotton soaked with saline. Stimulations were performed with monophasic, rectangular wave pulses of different voltages, rates and durations from a Grass S48 stimulator. An isolation unit SIU5 was connected between the stimulator and the animal. In preparatory experiments the voltage and current actually delivered to the nerve were determined with the aid of a resistance bridge, so that losses (especially in the SIU5) could be compensated for.

Immunocytochemical methods (VI)

Tissue specimens were immersed in an ice-cold buffered 4% formaldehyde solution overnight and rinsed in Tyrode solution containing 10% sucrose at 4°C for 48 hours, frozen on dry ice, and sectioned (15 µ) in a cryostat at -20°C.

Additional specimens from the maxillary nerve and sinus mucosa were fixed overnight in the same fixation as above. They were then immersed in a Tyrode solution containing 10% sucrose for 48 hours, briefly rinsed in a phosphate buffer and stretched on chrome-alum subbed microscope slides as whole mounts. Cryostat sections and whole mounts were processed for the immunocytochemical demonstration of SP using the indirect immunofluorescence methods of Coons et al (1955). A monoclonal antibody against SP (code no. NC1 34) from Sera-lab, UK, was used in a dilution of 1:80 (cryostat sections) or a dilution of 1:40 (whole mounts). The site of the antigen-antibody reactions was revealed by fluorescein isothiocyanate-labelled goat anti-mouse IgG diluted 1:20. Sections incubated with antibody inactivated by the addition of SP were used as controls.

Cigarette smoke exposure (VII)

Smoke was drawn with a syringe (volume 20 ml) from a commercial cigarette (Pall Mall, a king-size cigarette without filter). According to the manufacturer the smoke from one cigarette contains about 1.5 mg nicotine, 21 mg tar and 13 mg carbon monoxide. The cigarettes were smoked in a fume cupboard to a butt length of 25 mm and the smoke was administered as puffs of 2.5 ml once a minute (in one experimental series one puff every other minute) through a polyethylene catheter (inner diameter 1.5 mm) with its tip 2-3 mm in front of one of the nostrils. Most rabbits were exposed to the smoke from 40 to 25 mm butt length, sufficient for 3-4 consecutive puffs. The animals breathed room air between the challenges.

Calculations of results and statistical treatments

All experiments were started by recording the m.c. activity once a min until the wave frequency had been stable for approximately 30 min. During the different challenges continuous recordings were made on the ink writer, and the frequency calculated every 10 sec. The induced frequency changes were expressed as percentages of m.c. wave frequency (frequency zero level) immediately preceding a challenge. The mean value of the frequency variations during the 5 min preceding a stimulation was also calculated (baseline level) and compared with the frequency zero level and with the final level 10 min after administration. Agreement between these levels indicated that the preparation had remained stable between the different challenges.

The maximum induced frequency change (whether positive or negative) of a challenge expressed as a percentage of the frequency zero level was used for statistical evaluation.

The statistical method applied throughout was the analysis of variance. One-way analysis of variance was used for independent data, and two-way analysis of variance for dependent samples. An advantage of this method over the more commonly used t-tests is that several factors (treatments, dose levels etc) can be compared at the same time. Furthermore, a more efficient analysis is possible for

dependent samples since two-way analysis of variance allows comparison not only between treatments (columns) but also between different individuals (rows). The response to challenges between rabbits demonstrated obvious variations, whereas repeated challenges in the same rabbit (i.e. dependent samples) gave more uniform results. Therefore, each rabbit served as its own control whenever possible.

Analysis of variance (and t-tests) presupposes that the material conforms to the Gaussian (normal) distribution. The distributions of the m.c. responses to saline and SP challenges were analyzed, including the calculations of moments for skewness and kurtosis, and were found to be reasonably consistent with an assumed Gaussian distribution. However, the responses to experiments with nerve stimulation (V) were obviously not and the nonparametric tests Mann-Whitney U-test (independent samples) and the randomization test for matched pairs (dependent samples) were used instead (Siegel 1956).

The null hypothesis has been rejected throughout if $p<0.05$. Only two-tailed tests have been used.

Estimation of the experimental error of the method

Duplicate injections of 0.1 µg/kg SP and 0.5 µg/kg methacholine were performed in 10 rabbits with a time-interval of 10 min between challenges in order to estimate the experimental error. The doses were selected from the middle of the dose-response curves of SP and methacholine, and were used in several papers as reference injections. At least 30 min passed before experiments with the other compound started. The results are shown in Table I.

TABLE I

EXPERIMENTAL ERROR OF THE METHOD
Results of duplicate challenges in 10 rabbits

	First injection	Second injection	Experimental error
0.1 µg/kg SP	32.5 ± 4.1%	32.4 ± 3.3%	± 5.3%
0.5 µg/kg Metacholine	24.9 ± 1.5%	24.2 ± 1.8%	± 4.1%

Results are maximum frequency changes as percentages of the frequency zero level. Values are means ± SEM. Experimental errors are absolute values. Calculations of the experimental errors were made according to Rerup (1979). A complete analysis of the experimental error would require duplicate experiments over the entire dose-response ranges, which however seems unnecessary, since the method is not used as a bio-assay.

The Present Investigations

EFFECTS OF NEUROPEPTIDES ON MUCOCILIARY ACTIVITY (I).

Pharmacological and electrophysiological experiments have indicated that the autonomic nervous system influences m.c. activity. During recent years an increasing number of peptides have been identified in neuronal structures and the co-existence of a peptide and a classical transmitter has been demonstrated in several cases. As several peptides are found in the autonomic nervous system, and in sensory nerve fibres as well, there is reason to assume that they may influence m.c. activity.

The occurrence and distribution of neuropeptides in the upper airways has been mapped out by immunocytochemical methods and presence of VIP (vasoactive intestinal polypeptide), enkephalins, GRP (gastrin releasing peptide) and SP (substance P) has been confirmed. The purpose of the investigation was to study how these peptides influenced m.c. activity in the rabbit maxillary sinus and whether an evoked effect was modified by atropine pretreatment.

Results

VIP and enkephalins did not influence m.c. activity in the tested dose range 0.001-10.0 μg/kg. Bombesin (an amphibian peptide closely related to GRP) evoked a modest increase of m.c. activity (8.5-14.2%) at doses of 0.1-10.0 μg/kg.

SP increased m.c. activity in a dose-dependent manner clearly noticeable in the dose-interval 0.01-10.0 μg/kg (corresponding to 7.4 picomol-7.4 nanomol/kg), the maximum increase being about 50%. The SP-induced effect was characterized by a short latency with a peak response within 1-2 min and a duration of 2-5 min (Fig. 3). There was no indication of a late influence on m.c. activity after exposure to SP. Pretreatment with the anticholinergic drug atropine (0.2 mg/kg), which inhibits muscarinic receptors, did not affect the SP-induced acceleration. Contralateral injection of SP resulted in a significantly reduced and delayed response compared to ipsilateral injection in the same animal.

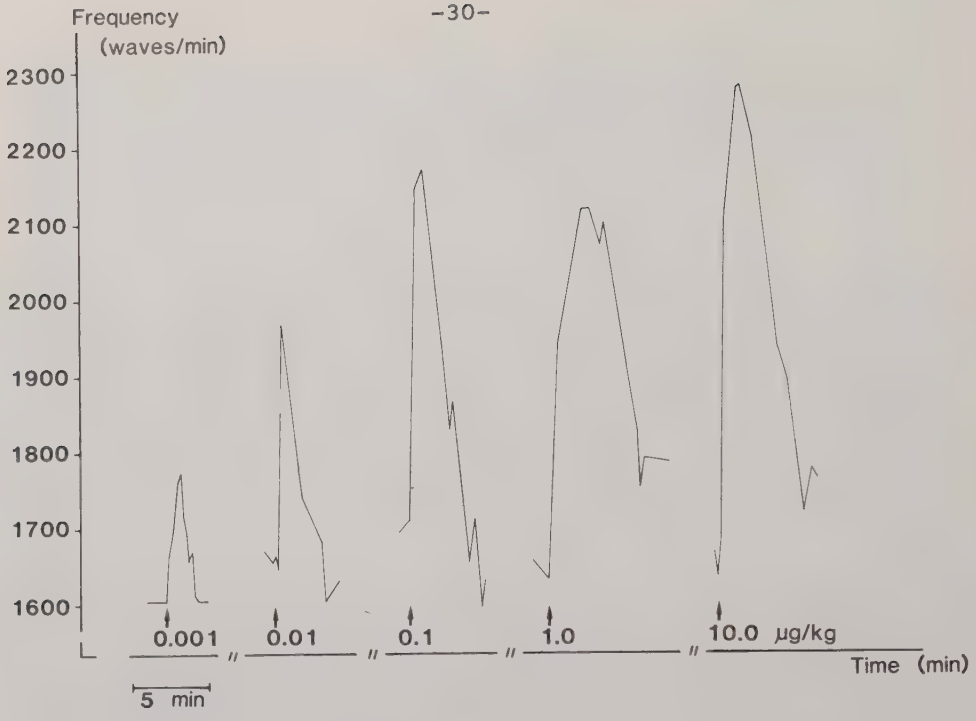

Fig. 3. The effect on the m.c. wave frequency in one rabbit after intraarterial injections of SP in the dose range 0.001-10.0 µg/kg SP (corresponding to 0.74 picomol-7.4 nanomol/kg).

Conclusions

VIP and enkephalins have no direct effect on m.c. activity. The weak response to bombesin, seen only after large doses, makes it doubtful whether the effect is of any physiological significance. In contrast to these findings, the accelerating effect of SP is equal to the effect of the parasympathomimetic drug methacholine on the same preparation. The effect of SP is probably direct, since atropine had no effect on the response. Moreover, the results of contralateral injections indicate that the mechanism through which SP induces its m.c. action is peripheral, further supporting the conclusion of a direct effect by SP.

SUBSTANCE P ANTAGONISTS AND MUCOCILIARY ACTIVITY IN RABBIT (II).

There is considerable evidence supporting the view that SP, or related peptides, in unmyelinated sensory C-fibres mediates the neurogenic inflammation produced by antidromic stimulation of peripheral nerve endings. A physiological role for SP in the respiratory tract has been reported. It has been shown that SP mediates protective mechanisms such as vasodilation and plasma extravasation. The acceleration of m.c. activity induced by exogenous SP might constitute a similar protective mechanism.

The role for SP in the regulation of m.c. activity can be elucidated by using SP antagonists. A number of SP antagonists are available, all being analogs of SP or truncated SP with D-amino acid substitutions. The purpose of the study was to investigate if SP antagonists inhibit the effect of SP on m.c. activity in rabbit maxillary sinus and, if possible, to select one which had a distinct and reversible blocking effect. This selected blocker was then used to antagonize m.c. responses to C-fibre stimulation by bradykinin or capsaicin.

Results

One SP antagonist, (Arg^{5}, D-Trp^{7,9}, Nle^{11})SP$_{5-11}$, stimulated m.c. activity, the maximal effect being 28.2 ± 5.5%, and was therefore abandoned. Two others, (D-Pro^{2}, D-Trp^{7,9})SP and (D-Arg^{1}, D-Trp^{7,9}, Leu^{11})SP ('Spantide'), were without effect per se and they were subjected to further investigations which showed that 1 mg/kg (D-Pro^{2}, D-Trp^{7,9})SP inhibited the m.c. response to 0.1 µg/kg SP whereas 'Spantide' had no apparent inhibiting effect. The responses to bradykinin and capsaicin were inhibited by (D-Pro^{2}, D-Trp^{7,9})SP. Methacholine 0.5 µg/kg stimulated m.c. activity irrespective of SP-receptor blockade by (D-Pro^{2}, D-Trp^{7,9})SP.

Conclusions

The results indicate that (D-Pro^{2}, D-Trp^{7,9})SP is an effective antagonist of the m.c. effect of exogenous SP, as well as of effects induced by bradykinin or capsaicin. Thus, (D-Pro^{2}, D-Trp^{7,9})SP emerges as a useful pharmacological tool for studying the role of SP in the control of m.c. activity in rabbit maxillary sinus.

Possible explanations for the difference between 'Spantide' and (D-Pro^{2}, D-Trp^{7,9})SP are that the latter may have a longer in vivo half-life or better bioavailability than 'Spantide', or that the antagonists may have different effects on different putative SP-receptors.

BRADYKININ ACCELERATES MUCOCILIARY ACTIVITY IN RABBIT MAXILLARY SINUS (III).

The basic neuropeptide SP has been put forward as a mediator of neurogenic inflammation in general. Certainly, SP seems to mediate protective mechanisms in the airway mucosa including vasodilation, plasma extravasation and acceleration of m.c. activity.

Another basic peptide thought to be involved in inflammatory reactions is bradykinin. Occurrence of bradykinin in nasal secretions has been proposed. Previous investigations regarding the biological effects of bradykinin suggest that bradykinin might release SP in the rabbit eye and increase activity in bronchial C-fibres. Thus, bradykinin can be expected to influence m.c. activity through release of SP from C-fibre endings. However, bradykinin is also a potent stimulator of prostaglandin synthesis, and some prostaglandins stimulate m.c. activity.

The purpose of the investigation was to study the effects of bradykinin on the m.c. activity in rabbit maxillary sinus. The mode of action of the drug was evaluated by pretreatment with the previous selected SP antagonist (D-Pro^2, D-Trp^{7,9})SP, by blockade of muscarinic receptors with atropine and by inhibition of prostaglandin synthesis with indomethacin.

Results

Bradykinin accelerated m.c. activity in the dose range 0.05-10.0 µg/kg (corresponding to 47 picomol-9.4 nanomol/kg). The increase reached maximum levels within one min, the activity still remaining elevated over the basal level after 5 min. Neither atropine nor indomethacin influenced the peak response to bradykinin. However, atropine pretreatment inhibited the initial phase of the response, whereas indomethacin was without apparent effect in this respect.

The SP antagonist (D-Pro^2, D-Trp^{7,9})SP inhibited the effect of bradykinin. The increase induced by 1.0 µg/kg bradykinin was reduced from 23.5 $\pm$ 3.0% to 5.3 $\pm$ 1.9%.

Conclusions

The results indicate that bradykinin accelerates m.c. activity through activation of neurons releasing SP, since the acceleration was inhibited by pretreatment with the SP antagonist (D-Pro^2, D-Trp^{7,9})SP.

Stimulation of prostaglandin synthesis seemed to be of less importance, since indomethacin did not affect the response to bradykinin. Atropine pretreatment suppressed the initial effect and the latency was prolonged, indicating that activity in cholinergic (parasympathetic) neurons could play an essential role in prompt acceleration of m.c. activity. This finding also suggests the existence of a reflex arc with SP-containing C-fibres as the afferent pathway and cholinergic neurons as the efferent pathway. The joint release of both SP and acetylcholine accelerates m.c. activity.

CAPSAICIN STIMULATES MUCOCILIARY ACTIVITY BY RELEASING SUBSTANCE P AND ACETYLCHOLINE (IV).

The question of whether the effect of exogenous SP on the m.c. system reflects physiological involvement of endogenous neuronal SP in the regulation of m.c. activity can be investigated by the use of SP-releasing drugs.

Capsaicin causes a release of SP-like immunoreactivity from central and peripheral sensory endings. Apart from effects related to SP release capsaicin applied locally induces hypertension, probably through sympathetic activation. It has also been reported that capsaicin initiates tracheal secretion through a reflex involving cholinergic effector neurons. Since both adrenergic and cholinergic compounds influence m.c. activity in the present experiment model, the effects of capsaicin need to be examined with respect to interactions with adrenergic and cholinergic pathways. A non-cholinergic, non-adrenergic effect of capsaicin on m.c. activity might be explained by release of SP. The SP antagonist ($D\text{-}Pro^2$, $D\text{-}Trp^{7,9}$)SP will inhibit such a response.

Furthermore, if reflex stimulation of the parasympathetic sphenopalatine ganglion is involved, the ganglionic blocker hexamethonium will inhibit the efferent component of such a reflex.

The aim of this investigation was therefore to study the acute effect of moderate doses of capsaicin on m.c. activity and the influence on this effect of adrenergic and cholinergic blockades, and ganglionic blockade. SP-receptors were blocked by the SP antagonist ($D\text{-}Pro^2$, $D\text{-}Trp^{7,9}$)SP.

Results

Capsaicin accelerated the m.c. wave frequency in the dose range 3.0-150.0 µg/kg (corresponding to 10.0 nanomol-0.5 µmol/kg), the maximum response being 40.3 $\pm$ 2.8% for a dose of 150.0 µg/kg. The response had a latency of 6.3 $\pm$ 0.9 sec to 30.0 µg/kg capsaicin (n=8). The peak frequency occurred within one min and the frequency returned to the basal level within a few min. Repeated challenges with capsaicin gave identical responses.

The effect of capsaicin was not affected by blockade of adrenergic nerves or of adrenoceptors. Neither guanethidine nor the combined pretreatment with propranolol and phentolamine influenced the increase of m.c. activity.

In contrast to this, atropine weakened the response to capsaicin, the maximum increases being halved. Analysis of the time-course for the effect of atropine showed that it inhibited the response to capsaicin during the first min after injection. The effect of capsaicin was also weakened by the ganglionic blocker hexamethonium. Like atropine, it reduced the maximum increases by about half. In contrast, neither atropine nor hexamethonium influenced the response to exogenous SP.

The SP antagonist (D-Pro^{2}, D-Trp^{7,9})SP almost abolished the response to capsaicin.

Conclusions

The effect of capsaicin on the m.c. system involves cholinergic, but not adrenergic pathways. The results indicate that C-fibres containing SP or SP-like peptides constitute the afferent pathway of a reflex arc in the airways with cholinergic effector neurons as the efferent pathway. Stimulation of this reflex arc causes an increase of m.c. activity through the joint release of SP from peripheral sensory nerve endings and of acetylcholine from cholinergic neurons.

ANTIDROMIC NERVE STIMULATION ACCELERATES MUCOCILIARY ACTIVITY IN RABBIT MAXILLARY SINUS (V).

The results of the previous pharmacological experiments indicated that release of endogenous SP, or SP-like peptides, can influence m.c. activity (III, IV). An alternative to pharmacological activation of C-fibres is electrical stimulation of peripheral sensory neurons. In the airways, this antidromic stimulation causes vasodilation and plasma extravasation probably as a consequence of the release of SP.

It is generally considered that high intensity stimulation of sensory nerves will evoke peripheral effects, attributable to SP release. A voltage of about 10 V is suitable. In contrast, low intensity stimulation (impulses of 0.5-2 V) can activate parasympathetic nerves to release acetylcholine.

Pharmacological activation of C-fibres indicates that part of the m.c. response is mediated by an atropine-sensitive (cholinergic) effect (III, IV). On the other hand, other reports have indicated that adrenergic influences may dominate in nasal circulation after sensory nerve stimulation.

The purpose of the study reported in (V) was therefore to investigate the effect on m.c. activity of antidromic nerve stimulation of the isolated maxillary nerve, which carries sensory nerve fibres from the maxillary sinus, and the influence on the response to pretreatments with atropine, the SP antagonist ($D\text{-}Pro^{2}$, $D\text{-}Trp^{7,9}$)SP and cervical sympathectomy.

Results

Low intensity stimulation of the maxillary nerve did not affect m.c. activity. Stimulation with impulses of 8 V, a pulse rate of 10 Hz and a pulse duration of 5 ms increased m.c. activity. The resulting acceleration in untreated rabbits amounted to 35.4 $\pm$ 4.9% (median 30.0%).

Pretreatment with atropine reduced the response to antidromic nerve stimulation especially during the first 2 min. The maximum increase was reduced to 20.0 $\pm$ 1.3% (median 21.3%). The SP antagonist abolished the response to antidromic nerve stimulation, the maximum increase being only 5.7 $\pm$ 1.3% (median 5.7%). In contrast, cervical sympathectomy did not influence the response to antidromic nerve stimulation.

Conclusions

Antidromic nerve stimulation accelerates m.c. activity, apparently by release of SP, or SP-like peptides. Reduction by atropine pretreatment indicates that part of the response is mediated by an effect on muscarinic receptors, via release of acetylcholine from as yet unidentified nerve fibres. However, the cholinergic part of the response is probably indirectly due to release of SP, since the SP antagonist abolishes the response and a low intensity stimulation,

known to release acetylcholine from parasympathetic neurons, is without effect.

The findings indicate that the interaction between SP neurons and parasympathetic cholinergic neurons in regulating m.c. activity is probably more complex than was expected from the previous results (III, IV). Not only might C-fibres constitute the afferent component of a reflex arc involving cholinergic neurons as the efferent pathway, there may also be a local reflex mediated via the sphenopalatine ganglion between neurons containing SP and neurons containing acetylcholine. Finally, direct stimulation of cholinergic neurons or release of acetylcholine from the maxillary nerve itself cannot be excluded.

THE MORPHOLOGICAL BASIS FOR THE EFFECT OF SUBSTANCE P ON MUCOCILIARY ACTIVITY IN RABBIT MAXILLARY SINUS (VI).

Morphological evidence for the existence of SP or SP-like peptides in the paranasal sinuses of any species has not been investigated. Previous conclusions (I-V) regarding the role of SP in the regulation of m.c. activity are based on the assumption that SP is present in the maxillary nerve and the maxillary sinus of the rabbit to essentially the same extent as has been found in the maxillary nerve and in the nose of other species. The aim of this investigation was therefore to study by immunocytochemistry the occurrence and distribution of SP fibres in the mucosa of the maxillary sinus, in the maxillary nerve and in the sphenopalatine ganglion of the rabbit. A monoclonal antibody directed against SP (NC1 34) was used.

Results

A rich supply of SP containing nerve fibres was found in the subepithelial layer of the maxillary mucosa in all rabbits studied. The fibres formed a network, and varicose endings were seen close to the ciliated epithelium (Fig 4).

Fig. 4. Rabbit maxillary sinus. Cross section of the mucous membrane showing subepithelial SP-immunoreactive nerve fibres (arrows) with varicosities close to the ciliated epithelium (EP). (X 225).

SP fibres were abundant in the maxillary nerve and the sphenopalatine ganglion, where fine beaded SP immunoreactive nerve fibres formed a network in the ganglion and frequently surrounded ganglion cells in a basket-like fashion.

Conclusions

The result confirms the presence of SP (or SP-like peptides since cross-reactivity to other tachykinins with a common C-terminal sequence to SP cannot be ruled out) in the maxillary sinus and favours the view that SP effects on m.c. activity are physiological and not merely pharmacological.

Furthermore, the existence of SP nerve endings and nerve fibres in the rabbit sphenopalatine ganglion provides a morphological basis for a local reflex arc, involving both SP neurons and parasympathetic effector neurons, which may be of importance in the regulation of m.c. activity.

REFLEX-INDUCED ACCELERATION OF MUCOCILIARY ACTIVITY AFTER SHORT-TERM EXPOSURE TO CIGARETTE SMOKE (VII).

Electrophysiological recordings of the activation of airway C-fibres have only been made from the lower airways. Such fibres are stimulated by endogenous mediators such as histamine, serotonin and prostaglandins and by several irritants.

Cigarette smoke is a common airway irritant. In contrast to the well known harmful effect on the respiratory epithelium (including the m.c. system) of long-term exposure to cigarette smoke, short-term exposure accelerates m.c. activity. Cigarette smoke not only increases m.c. activity in the present experiment model but also m.c. clearance in man. Previous results have indicated that exposure to cigarette smoke induces oedema in the nasal mucosa and nose wiping behaviour in experimental animals. This is probably due to stimulation of C-fibres containing SP, since both responses were inhibited by pretreatment with high doses of capsaicin or with an SP antagonist. Furthermore, experiments on dogs have indicated that bronchial C-fibres are activated by exposure to cigarette smoke. Thus, the m.c. response to short-term exposure to cigarette smoke may be a combined effect produced by SP released from the afferent pathway of the reflex arc (axon reflex) previously described (III, IV, V) and acetylcholine from the cholinergic effector pathway.

In order to analyze the roles played by SP and acetylcholine on m.c. activity after exposure to cigarette smoke, rabbits were pretreated with atropine, the SP antagonist (D-Pro^2, D-Trp^{7,9})SP, repeated high doses of capsaicin (in order to desensitize capsaicin-sensitive C-fibres) or the ganglionic blocker hexamethonium.

Results

Cigarette smoke increased m.c. activity by about 30%. The latency of the response was 3.7 $\pm$ 0.5 sec and it was also possible to evoke a response after challenge with smoke presented to the contralateral nostril.

All pretreatments suppressed the response to cigarette smoke. However, the duration of the suppressions after pretreatment with atropine and (D-Pro^2, D-Trp^{7,9})SP was short, and the effect of these drugs declined with each renewed exposure.

Rabbits which were adequately atropinized, as evidenced by the lack of response after challenge with the parasympathomimetic drug methacholine, still responded to cigarette smoke with an accelerated m.c. activity, albeit reduced. Repeated atropinizations in these rabbits gave the same type of response, but if the SP antagonist was added to the muscarinic blockade a more prolonged and stronger inhibition of the response to tobacco smoke was seen.

Conclusions

The increased m.c. activity noticed after short-term exposure to cigarette smoke is probably produced by a nervous reflex, since it is also evoked by smoke applied to the contralateral nostril.

The inhibition of the response after pretreatment with the SP antagonist or repeated doses of capsaicin demonstrates that C-fibre stimulation is essential for the accelerating effect of cigarette smoke on m.c. activity.

Pretreatment with atropine clearly weakened the response to cigarette smoke, indicating that part of it is mediated through cholinergic effector neurons. However, increased m.c. activity could still be observed in rabbits that did not respond to injections with methacholine. This non-cholinergic increase was inhibited by the addition of the SP antagonist. Taken together with the strong stimulating effect of SP on m.c. activity, this finding supports the view that cigarette smoke activates a reflex arc involving C-fibres containing SP, or SP-like peptides, as the afferent pathway and cholinergic effector neurons as the efferent pathway. The dual release of SP from the peripheral sensory endings (axon reflex) and acetylcholine from cholinergic (probably parasympathetic) neurons accelerates m.c. activity.

The finding that the ganglionic blocker hexamethonium inhibits the response to cigarette smoke does not necessarily contradict the hypothesis proposed above. In other experiment models hexamethonium has been found to suppress effects attributable to C-fibre activation suggesting that hexamethonium-sensitive nicotinic receptors may exist on peripheral branches of sensory nerves. Thus, hexamethonium not only blocks the efferent pathway of the reflex, but it probably suppresses the afferent component too.

General Discussion and Clinical Aspects

This thesis provides experimental evidence that SP or similar tachykinins released from unmyelinated C-fibres are involved in the regulation of m.c. activity in the rabbit maxillary sinus. The effects of C-fibre activation comprise direct stimulation of m.c. activity by release of SP and secondary mucosal release of acetylcholine from cholinergic effector neurons. This dual mechanism, which appears to be activated by irritants and inflammatory mediators, may be of importance in the defence of the airways particularly against inhaled toxic substances and other contaminants. However, there are some limitations in our present knowledge which deserve attention.

The method used in the present investigation provides information on m.c. activity but gives little information about the transport efficiency of the m.c. system. An increased m.c. wave frequency does not necessarily mean a more efficient transport of mucus. Although there is one report indicating a direct relation between the m.c. wave frequency and mucus flow rate (Hée & Guillerm 1977) this has not been confirmed in the rabbit maxillary sinus, nor for SP. The assumption that an increased m.c. wave frequency in the rabbit maxillary sinus also means improved m.c. transport is based on analogies between results obtained in the present experiment model and measurements of m.c. transport or clearance after challenges with pharmacological compounds (methacholine, β-agonists) or cigarette smoke (see page 21). Nevertheless, the effects of SP and C-fibre stimulation on mucus flow rate remain to be established.

Of importance is the question whether SP antagonists merely act as reversible competitive antagonists (Håkanson et al 1982, Rosell et al 1983, Folkers et al 1984) or whether they also induce functional changes in the C-fibres. A local anesthetic action of SP antagonists in the frog sciatic nerve has been reported (Karlsson et al 1984). Furthermore, thecal injections with SP antagonists produce paralysis and degeneration of spinal motor neurons in rats (Post et al 1985). The injected amount of the antagonists only slightly exceeded that producing antinociceptive effects in the same animals. On the other hand, other investigations have not demonstrated a local anesthetic effect of SP antagonists in mammals (Bynke et al 1984, Lundblad et al 1984). However, some findings in the present work that (D-Pro^{2}, D-Trp^{7,9})SP blocks not only non-cholinergic effects attributable to SP but also the expected effects of secondarily released acetylcholine after C-fibre stimulation with pharmacological challenges (III, IV), electrical stimulation (V) and cigarette smoke (VII) might suggest a complex action of SP antagonists.

One would expect a residual cholinergic response after C-fibre activation, since SP antagonists do not seem to penetrate the blood-brain barrier (R Håkanson, personal communication). Effects evoked by release of SP from the central endings of the C-fibres are probably therefore not antagonized by (D-Pro^2, D-Trp^{7,9})SP. Moreover, SP (and other tachykinins) are not the only putative transmitters in C-fibres. For example, it has been reported that another peptide CGRP co-exists with SP in capsaicin-sensitive neurons as evidenced from immunocytochemical studies (Lundberg et al 1985).

It is unlikely that the SP antagonists tested in the experiments reported here have had any local anesthetic effect. The known effect of local anesthetics is to decelerate m.c. activity (Dudley & Cherry 1978, Manawadu et al 1978), whereas the SP antagonists did not reduce m.c. activity per se. The unaltered response to methacholine during SP receptor blockade (II) further supports the view that the effect of (D-Pro^2, D-Trp^{7,9})SP was due to a specific SP receptor blockade, rather than to a nonspecific action of the drug. The effect of the SP antagonist was clearly reversible (II, III, IV, VII). Moreover, the effect of the SP antagonist with each successive exposure to cigarette smoke weakened faster (VII) than was expected from previous experiments with exogenous SP (II), suggesting displacement of the antagonist by endogenous SP. It was also noticed that the SP antagonist 'Spantide', which is more neurotoxic after thecal injections than (D-Pro^2, D-Trp^{7,9})SP (Post, personal communication), had a far smaller inhibiting effect than (D-Pro^2, D-Trp^{7,9})SP on the response to exogenous SP, when 1 mg/kg was given of both compounds (II). Thus, although functional changes in the C-fibres themselves could not be ruled out, there was clearly evidence that (D-Pro^2, D-Trp^{7,9})SP had a selective effect on the m.c. response.

In the skin, capsaicin-sensitive C-fibre receptors probably constitute polymodal nociceptors and heat receptors (Szolcsányi 1977, 1984). They are capable of releasing SP and are thought to be involved in neurogenic inflammatory responses (see Lembeck & Gamse 1982). The functional significance of C-fibres in the airways is probably also related to inflammation, in particular to the effects of irritants (Coleridge & Coleridge 1984) and they are involved in neurogenic protective mechanisms such as vasodilation (Lundblad et al 1983a) and plasma extravasation (Lundblad et al 1983c). The increase in m.c. activity is another protective mechanism related to the activation of C-fibres, probably through the release of SP. The peripheral release of SP takes place both from the stimulated nerve ending itself, and from collaterals (axon reflex) (see Szolcsányi 1984). The ostium of the maxillary sinus in rabbits is roughly 0.5x4 mm in cross section and 2.6 mm in depth (Kumlien & Schiratzki 1985). It is therefore unlikely that the epithelium of the maxillary sinus was in direct contact with cigarette smoke (VII). Thus, the response evoked in the maxillary sinus in

atropinized rabbits indicates that an axon reflex is involved in the m.c. response to cigarette smoke.

It has been suggested that SP released from C-fibre endings stimulates mast cells to release histamine (Lembeck & Holzer 1979, Lembeck & Gamse 1982). Histamine accelerates m.c. activity in the present experiment model, but the physiological significance of this observation is very doubtful, because doses of 0.1-1.0 mg/kg are required before any response is demonstrated (Lindberg & Mercke, unpublished observations). One may speculate that the acceleration of m.c. activity after challenge with histamine is in fact induced by unspecific C-fibre stimulation, since histamine is known to activate bronchial C-fibres (Coleridge et al 1978). Evidently, the interaction between C-fibre activation, SP release and inflammatory mediators needs further elucidation.

If SP is a transmitter in some sensory neurons, then central effects are to be expected as well. SP is thought to be involved in the transmission of pain (Henry et al 1980) and C-fibre activation is known to trigger protective reflexes in the airways, such as coughing and sneezing (Collier & Fuller 1984, Lundblad et al 1984). Exogenous SP is less likely to evoke these responses since the peptide probably does not pass the blood-brain barrier.

The rapid initial phase of the m.c. response to C-fibre activation was suppressed by atropine (III, IV, V) indicating that part of the m.c. acceleration involved release of acetylcholine. The nerve fibres releasing this acetylcholine are not clearly identified, but the hypothesis includes above all parasympathetic effector neurons. This view is supported by the suppression of the capsaicin-induced response by the ganglionic blocker hexamethonium (IV). Moreover, Davis et al (1982) reported atropine-sensitive reflex secretion from canine tracheal glands after challenging bronchial circulation with bradykinin or capsaicin i.a. Previous electrophysiological experiments had demonstrated that similar doses of these compounds selectively stimulate bronchial C-fibres (Coleridge & Coleridge 1977, Kaufman et al 1980). The similarities of the responses reported in the present study and in the previous investigation in the lower airways in another species confirm the existence of a reflex.

The connections between afferent C-fibres and efferent parasympathetic neurons might be in the brainstem, or in the sphenopalatine ganglion, or both. A central reflex arc is indicated by the m.c. response to contralateral challenges with tobacco smoke (VII). On the other hand, the finding of a cholinergic component in the response to antidromic nerve stimulation (V) of the isolated and severed maxillary nerve suggests the existence of peripheral connections between C-fibres and cholinergic neurons. A morphological basis for this latter point of view is to be found in the SP nerve endings observed close to the ganglion cells in the sphenopalatine ganglion (VI). The finding that D-Pro^{2}, D-Trp^{7,9})SP

abolishes the response to C-fibre stimulation (III, IV, V, VII) further supports the existence of a peripheral reflex, since SP receptors in the sphenopalatine ganglion are in all probability blocked by infusion of the antagonist. In contrast to this, the observation that the m.c. response to exogenous SP is resistant to atropine pretreatment (I, IV) does not support the hypothesis of a local connection. Still another possible point of interaction between SP and acetylcholine is suggested by the fact that sensory trigeminal neurons stain for acetylcholinesterase, including the SP neurons themselves (Tervo et al 1983). Acetylcholine may thus be released from the sensory nerve endings themselves together with SP in addition to the reflex release of acetylcholine. One must bear in mind that another as yet unknown mediator in C-fibres might be responsible for the cholinergic part of the m.c. effect of C-fibre activation. This possibility is independent of whether connections between C-fibres and cholinergic effector neurons occur at a central or peripheral level. However, the effect of the SP antagonist does not support the view that other mediators than tachykinins are involved in the reflex release of acetylcholine. Some of the hypothetical mechanisms regulating m.c. activity discussed in the present thesis are summarized in Fig. 5.

The reflex cholinergic response could be further evaluated by severing the preganglionic parasympathetic nerve (the Vidian nerve) or removing the sphenopalatine ganglion. However, such procedures have so far been impossible in the rabbit without interfering with the sensory innervation to the maxillary sinus.

The effects on the m.c. system of SP released from unmyelinated C-fibres are not known in detail. However, it seems likely that SP induces secretory changes since *in vitro* experiments with secretory cells from the canine airways have shown SP to have a strong stimulatory effect on glycoprotein production (Baker et al 1977). Furthermore, SP increases net Cl^- secretion towards the lumen from the canine trachea and elicits a rapid rise in short circuit current within 3-5 sec of administration despite pretreatment of tissues with atropine or adrenergic antagonists (Al-Bazzaz et al 1985), indicating that nerve fibres containing SP may play a role in the regulation of ion transport across the trachea. Net movements of Cl^- towards the lumen will increase the water content of the respiratory secretions and in turn affect m.c. activity (Nadel & Davis 1978). SP has little or no effect, on the beating of the cilia in explants from human adenoids, guinea pig trachea or rabbit trachea and maxillary sinus (Khan & Lindberg, unpublished results). Thus, there is probably no effect of SP on the cilia themselves. Instead, it is likely that the m.c. response to SP *in vivo* represents a rapid change in the periciliary fluid, causing acceleration of m.c. activity.

The periciliary layer is probably produced by the epithelial cells themselves (see page 11) and increased secretion, not only from submucosal glands but also from the epithelial

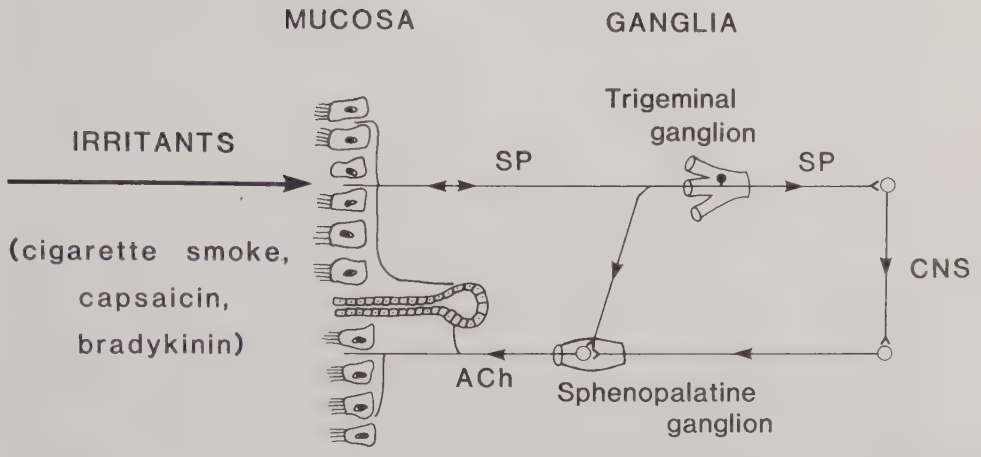

Fig. 5. Model for the protective reflex regulating m.c. activity studied in the present thesis. C-fibre endings in the epithelial and subepithelial layer are stimulated by irritants such as cigarette smoke (VII), and by bradykinin or capsaicin (III, IV). M.c. activity is accelerated by SP released from these fibres and by acetylcholine (ACh) released from cholinergic (parasympathetic) effector neurons. C-fibre afferents connect with cholinergic efferents via the brainstem and possibly via the sphenopalatine ganglion as well. Other possibilities for connections between SP and ACh such as direct stimulation of cholinergic effector neurons or the occurrence of ACh in the sensory endings themselves have been omitted. Arrows ⟶ indicate the direction of impulse propagation. ⟷ symbolizes the dual sensory-efferent function of C-fibre afferents. Note that C-fibres also contain at least two other peptides SK and CGRP besides SP and that parasympathetic neurons contain VIP and PHI as well.

= ciliated cell = submucosal gland

The drawing is a modification of an illustration in Lundblad (1984).

lining, might help the mucosal membrane to entrap and possibly dilute inhaled irritants and other contaminations. In this context, it seems likely that SP is one of the local mediators (proposed by Phipps 1980) of the non-cholinergic, non-adrenergic secretory response to exposure to irritants. Interestingly, C-fibres could also constitute the afferent pathway of the reflex described by Phipps (1980), that regulates the secretory response to irritants in cat trachea, since inhalation of capsaicin clearly stimulates cough receptors (Collier & Fuller 1984). However, further investigations are needed before the question whether SP and C-fibres also mediate the m.c. response to other irritants than cigarette smoke (VII) can be settled.

Some reports suggest the existence of a species difference in the response to SP in the airways. Lundberg et al (1983b) have reported that human bronchi responded less well to SP than to acetylcholine or histamine. In contrast, SP and capsaicin were much more potent in guinea-pig trachea. This reflects either a species specific difference, or the fact that the human bronchi were taken from elderly patients with a history of smoking and bronchitis. Further, SP provokes nasal secretion in the dog and the rat but not in man, although general symptoms of vasodilation appear (Pettersson & Malm, unpublished observations). It remains an open question whether such results make it difficult to generalize from the present findings to the regulation of m.c. activity in man. On the other hand, SP is found in the human nose (Uddman et al 1983) and studies on human skin (Jancso et al 1968, Bernstein et al 1981) and airways (Collier & Fuller 1984) indicate that SP and other tachykinins have physiological effects also in man.

Clinical aspects

Several common respiratory conditions in the airways are associated with impaired m.c. activity, e.g. otitis media with effusion and chronic sinusitis in the upper airways, and chronic bronchitis, cystic fibrosis and asthma in the lower airways (see Phipps 1981). The current treatment for these conditions in the upper airways based on nasal decongestants (mainly nose drops or aerosol sprays), is not without drawbacks. The α-agonists used seem to have dubious effects on the m.c. system (Simon et al 1977, Hybbinette & Mercke 1982c). In addition, there is a toxic effect from the preservatives in nose drops (Van de Donk et al 1980, Håkansson & Toremalm 1983). In fact, it has been claimed that decongestants contribute to the etiology of otitis media with effusion (Peerless & Noiman 1980).

On the other hand, the β_2-agonists used in the therapy of obstructive and hyperreactive lung disorders stimulate m.c. function (Santa Cruz et al 1974, Camner et al 1976, Hybbinette & Mercke 1982c). Mucus flow rate is also increased by methylxanthines (Serafini et al 1976, Sutton et al 1981).

The findings of the present investigations may offer new approaches in the search for treatments of impaired m.c. function. The acceleration of m.c. activity induced by release of SP, or SP-like peptides, from sensory nerve endings was probably always associated with a simultaneous release of acetylcholine (III, IV, V, VII). Stimulation of cholinergic nerves is known to increase m.c. transport (Slaughter & Aiello 1982, Morley & Sanjar 1984). This protective reflex mechanism in the airways is presumably activated under many different conditions. C-fibre receptors in the lung are stimulated by inhaled irritants and endogenous mediators such as histamine, bradykinin and prostaglandins (see Coleridge & Coleridge 1984). In other words, C-fibre endings seem to be stimulated by any lesion in the airway mucosa. For example, vagal C-fibres are more sensitive during experimental pneumonia in cats

(Frankstein & Sergeeva 1966). It has been observed that the firing rate of C-fibres in the dog lung is increased by deep inflations (Kaufman et al 1982). Increased m.c. activity during infection and other inflammatory conditions is beneficial in a teleological sense, and pharmacological or physiotherapeutical manipulation of this reflex may be of therapeutic value.

It must be emphasized that the events studied in the present work all have a short duration (for example the response to cigarette smoke in paper VII). It is not known whether prolonged C-fibre activation increases secretions to such an extent that m.c. activity (and mucus flow rate) is in fact impaired in the long run. However, the sinus never became blocked by mucus during the experiments with SP. In contrast, challenges with methacholine generally produced a noticeable increase in the volumes of the secretions. One may speculate that prolonged exposure to irritants might tend to exhaust the m.c. system and desensitize the C-fibres (a parallel to the effect of capsaicin). The end-result would then turn out to be impaired m.c. function including impaired responsiveness of the m.c. system (see Lippmann & Schlesinger 1984).

Indirect support for the importance of the studied reflex mechanism is provided by Gamse and co-workers (1981) who gave very high doses of capsaicin to neonatal rats in order to deplete SP. They reported that most of the rats that died after this treatment (the mortality was about 50% within the first 3-5 weeks), showed extensive atelectatic areas in the lungs, a condition which is associated with impaired m.c. function, and accompanying stagnation of mucus plugs.

General Summary

A protective reflex in the rabbit maxillary sinus is presented. This reflex comprises unmyelinated C-fibres (containing SP or similar tachykinins) as the afferent component and cholinergic (parasympathetic) effector neurons as the efferent component. The reflex regulates m.c. activity in the rabbit maxillary sinus after C-fibre stimulation by release of SP or SP-like peptides from the peripheral sensory endings of the reflex arc and by release of acetylcholine. This concept is supported by the following experimental evidence:

1. SP, or SP-like peptides, is present in nerve fibres showing varicosities close to the ciliated epithelium in the rabbit maxillary sinus, in the rabbit maxillary nerve and in nerve fibres in the rabbit sphenopalatine ganglion (VI).

2. Exogenous SP accelerates m.c. activity in the rabbit maxillary sinus. The effect is not mediated via muscarinic receptors and it is not influenced by ganglionic blockade (I, IV).

3. The effect of exogenous SP is blocked by the SP antagonist (D-Pro^2, D-Trp^{7,9})SP. The blockade is reversible and does not involve muscarinic receptors (II).

4. The effect of SP on the m.c. system is mimicked by pharmacological activation of C-fibres by bradykinin or capsaicin. The effect of these drugs is abolished by pretreatment with the SP antagonist (D-Pro^2, D-Trp^{7,9})SP (III, IV).

5. Antidromic electrical stimulation of the maxillary nerve, at intensities known to stimulate C-fibres, results in an increase of m.c. activity that is abolished by pretreatment with the SP antagonist (D-Pro^2, D-Trp^{7,9})SP. The effect of antidromic nerve stimulation is not influenced by previous cervical sympathectomy (V).

6. In contrast to the effect of exogenous SP, pretreatment with atropine suppresses the response to both pharmacological and electrical C-fibre stimulation (III, IV, V). This finding suggests that unmyelinated C-fibres constitute the afferent component of a reflex arc with cholinergic effector neurons as the efferent pathway. The reflex arc may involve connections at different levels, such as the brainstem or the sphenopalatine ganglion (V, VI, VII).

7. Short-term exposure to cigarette smoke accelerates m.c. activity. The increase is probably mediated by triggering the above-mentioned reflex, since the response is not only suppressed by atropine but also by the SP antagonist (D-Pro^{2}, D-Trp^{7,9})SP and by pretreatment with repeated doses of capsaicin. Furthermore, the non-cholinergic response to cigarette smoke is inhibited by pretreatment with the SP antagonist (D-Pro^{2}, D-Trp^{7,9})SP (VII).

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EFFECTS OF NEUROPEPTIDES ON MUCOCILIARY ACTIVITY

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Abstract

The effects of four neuropeptides, vasoactive intestinal polypeptide, enkephalin, bombesin and substance P, on mucociliary activity in the rabbit maxillary sinus were investigated in vivo. The peptides were administered via the feeding artery (a. maxillaris), and the resulting effects were registered with a non-invasive photoelectric technique. The peptides were tested in the dose range 0.0001-10.0 µg/kg body weight. The following results were observed:

1. Vasoactive intestinal polypeptide (VIP) and enkephalin did not influence mucociliary activity.

2. Bombesin had only a slight accelerating effect on the mucociliary activity at doses of 0.1-10.0 µg/kg.

3. Substance P (SP) markedly accelerated the mucociliary activity in a dose-dependent manner in the dose range 0.01-10.0 µg/kg, the maximal increase being about 50%. The effect of SP was atropine-resistant and probably exerted directly on the mucosa.

Introduction

The factors influencing ciliary beat frequency and coordination have not been satisfactorily identified (1). Pharmacological experiments have indicated an influence of the autonomic nervous system on mucociliary activity. Hybbinette & Mercke (2) found that the cholinergic (parasympathetic) agonist methacholine caused a powerful and dose-dependent acceleration of mucociliary wave frequency in the rabbit maxillary sinus, whereas the cholinergic antagonist atropine had no influence on the basal mucociliary activity. Several authors have reported similar results using different in vivo and in vitro test models (3,4,5,6). The sympathetic nervous system is probably of less importance, although β_2-stimulators (salbutamol, terbutaline) increase mucociliary activity and transport (6,7,8).

During recent decades an increasing number of peptides have been identified in neuronal structures, including parasympathetic neurons, and the coexistence of a peptide and a classical transmitter has been demonstrated in several cases (9). A putative transmitter role has been substantiated for some neuropeptides, while for others experimental evidence is lacking. As several peptides are found in the autonomic nervous system, they may influence mucociliary activity either as modulators or as transmitters.

The occurrence of neuropeptides in the upper airways has been mapped out by means of immunohistochemistry, e.g. vasoactive intestinal polypeptide (VIP), enkephalins, gastrin releasing peptide (GRP) and substance P (SP).

VIP is found in nerve fibres in the nasal mucosa, particularly surrounding small blood vessels and seromucous glands (10). VIP is also present in the pterygopalatine ganglion, probably in the same cells as acetylcholine (11, 12).

Enkephalins, together with VIP and SP, have been demonstrated in the middle ear mucosa and eardrum of the cat and guinea pig (13).

GRP has been demonstrated in the nasal mucosa, the middle ear and in the tracheobronchial walls (14). GRP probably represents the mammalian counterpart of the amphibian peptide bombesin. They have the C-terminal 7 amino acids in common, and have similar effects on smooth muscle preparations (15,16).

SP, which was discovered by von Euler and Gaddum 1931 (17), is found in neurons in the trigeminal ganglion and in nerve fibres terminating in the subepithelial layer of the nasal

mucosa, sometimes penetrating the epithelium (11,18,19).

However, no information concerning the effect of these above-mentioned neuropeptides on mucociliary activity is available. The aim of the present investigation has therefore been to study:

1. The effect of VIP, enkephalin, bombesin and SP on mucociliary activity.

2. Whether an effect evoked by these neuropeptides is influenced by atropine blockade, and

3. Whether an effect produced by these neuropeptides is exerted directly on the mucosa or mediated by central action.

Materials and Methods

The experiments were performed on 40 male rabbits of a white strain, raised under controlled conditions and weighing 1.8-2.8 kg. Details of anesthetic and surgical techniques have been described previously (20). The animals were anesthetized with urethane i.m. 2 g/kg as an initial dose, and an extra dose of 0.5 g/kg was given in an ear vein during the operation. The facial artery or lingual artery was cannulated, and the cannula was continuously perfused with physiological saline 2-5 ml/h in order to keep the inlet open. This intraarterial route was used for administering the drugs. The mucosa was exposed via a trepanation hole of about 2x8 mm, which was immediately sealed with a window and bone wax (Ethicon). The mucociliary activity was recorded from the frontosuperior part of the maxillary sinus using a photoelectric technique. This technique has been described in detail by Mercke et al in 1974 (21) and its validity has been tested and confirmed both in model experiments and in biological investigations (22, 23, 24). A light beam is aimed at a ciliated mucous membrane so that a light reflection is created. The intensity of the reflected light is constantly changing as the movements of the cilia give rise to a pattern of wave movements in the covering mucus layer. The variations of light intensity are a measure of the frequency of wave movements, i.e. the mucociliary activity of the mucous membrane. Using a phototransducer the optical signals are transformed into electrical voltages. The mucociliary activity can then be monitored continuously on an oscilloscope and be recorded on an inkwriter. In the present investigation the recordings were analyzed both manually and with the aid of a computerized frequency calculator. The mucociliary activity was expressed in waves/min. The induced frequency changes were expressed as percentages of the mucociliary wave frequency at the time of administration of the test substances (=frequency zero level). The mucociliary wave frequency was calculated automatically every 10 sec during the experiments, and at intervals of 1-5 min otherwise. ECG and rectal temperature were monitored, and body temperature was adjusted to normal (i.e. 37.0-38.5^{o}C) with the aid of a heating pad.

The following drugs were tested: VIP, leu-enkephalin, met-enkephalin, bombesin and SP (Beckman Bioproducts, Geneva, Switzerland). The peptides were dissolved in physiological saline containing 0.5% albumin (Kabi, Sweden) in order to prevent the peptides from adhering to the walls of glass and plastic cannulas. All polystyrene material was rejected for the same reason. Atropine sulphate (ACO, Sweden) was used to block muscarinic receptors.

Experimental procedure: Initially, the mucociliary activity was recorded every min until the frequency had remained stable for approximately 30 min. In addition the mean value of the frequency variations during the last 5 min preceding an injection (=baseline level) were compared with the frequency zero level, as well as the final level 10 min after administration of a drug. The test solutions were delivered as bolus doses of 0.2 ml during 3 sec in retrograde direction through the cannula. Saline injections (with and without albumin) served as controls and did not influence the mucociliary activity. The time lapse between renewed injections of peptides was at least 10 min. If mucociliary activity was influenced, the time lapse was increased to 15-20 min. All peptides were tested in the dose range 0.0001 (0.1 ng) - 10.0 µg/kg.

The dose-response curves were usually established starting with the lowest dose first. However, in some animals the procedure was reversed, starting with the highest dose. All dose-response results were obtained in animals, who had not been challenged with another drug before. Atropine sulphate was given intra-arterially in a dose of 200 µg/kg 2 min before the injection of SP, which is sufficient to inhibit the effect of the parasympathomimetic drug methacholine on the same preparation (2).

In experiments designed to compare the effects of ipsilateral and contralateral injections of SP the contralateral facial or lingual artery was also cannulated.

The results were evaluated statistically by analysis of variance.

Results

VIP (54 experiments in 9 rabbits) did not influence mucociliary activity in the dose range 0.0001-10.0 µg/kg. The slight acceleration is within the normal variability of the method (Fig. 1). Thus the effect of VIP did not significantly differ from the effect of the saline controls.

Leu-enkephalin (30 experiments in 5 rabbits) did not influence mucociliary activity in the dose range 0.0001-10.0 µg/kg. As for VIP the increase in mucociliary wave frequency is within the variability of the method (Fig. 2). The results of the met-enkephalin experiments (27 experiments in 3 rabbits) did not differ from the leu-enkephalin experiments.

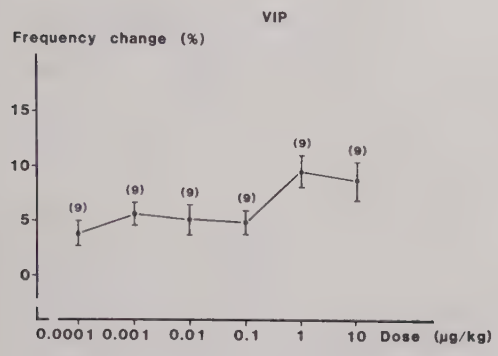

Fig. 1. The effect on mucociliary activity of VIP intraarterially in the dose range 0.0001 - 10.0 µg/kg. Figures in brackets refer to the number of experiments performed. The results are expressed as means and standard error of the mean (SEM) (n=9). Frequency zero level was 1358 ± 250 waves/min (mean ± SD).

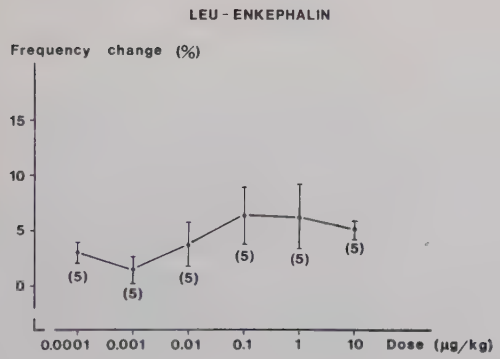

Fig. 2. The effect on mucociliary activity of leucine-enkephalin intraarterially in the dose range 0.0001 - 10.0 µg/kg. Figures in brackets refer to the number of experiments performed. The results are expressed as means and SEM (n=5). Frequency zero level was 1399 ± 198 waves/min (mean ± SD).

Bombesin (36 experiments in 7 rabbits) accelerated mucociliary wave frequency at doses of 0.1-10.0 µg/kg. The increase was modest (8.5-14.2%) but of statistical significance ($p<0.05$) (Fig. 3).

Injection of SP accelerated the mucociliary activity in a dose--dependent manner. The effect was clearly noticeable in the dose interval 0.01-10.0 µg/kg (corresponding to 7.4 picomol to 7.4 nanomol/kg), the maximum increase being about 50%. The dose-response relationship is illustrated in Fig. 4, which is based on the results obtained from 77 experiments in 14 rabbits ($p<0.001$).

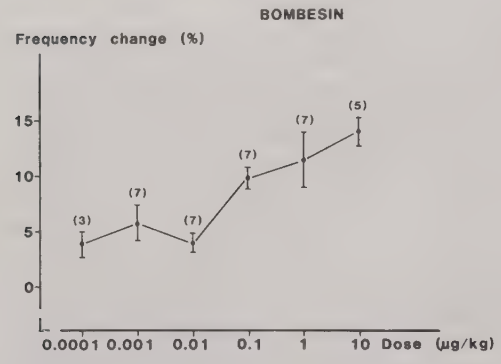

Fig. 3. The effect on mucociliary activity of bombesin intraarterially in the dose range 0.0001 - 10.0 µg/kg. Figures in brackets refer to the number of experiments performed. The results are expressed as means and SEM (n=7). Frequency zero level was 1344 ± 282 waves/min (mean ± SD).

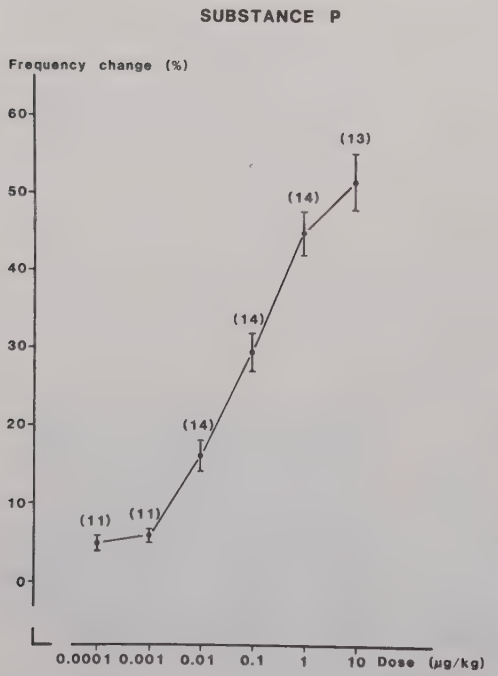

Fig. 4. The effect on mucociliary activity of SP intraarterially in the dose range 0.0001 - 10.0 µg/kg. Figures in brackets refer to the number of experiments performed. The results are expressed as means and SEM. The correlation coefficient for raw data: r=0.81 (n=14). Frequency zero level was 1398 ± 241 waves/min (mean ± SD).

Some of the underlying data illustrated in Fig. 4 are explained in detail in Table I, which shows the results of 0.01-10.0 µg/kg SP given to 7 of the rabbits. Table I emphasizes that there was no indication of a late influence on mucociliary activity after exposure to SP, since the mucociliary wave frequency always returned to baseline levels. Thus the preparation remained unchanged after drug administration. It is also evident from Table I that the spontaneous variations in mucociliary activity were of minor importance to the present results, despite the differences in baseline and zero frequency levels between individuals.

The SP-induced effect is characterized by a short latency with a peak response within 1-2 min and a duration of 2-10 min. The higher the dose given, the longer the duration of the effect. Figure 5 shows in chronological order the results of 5 experiments with different doses on one rabbit. The responses were reproducible irrespective of whether increasing or decreasing doses were given. Reproducible responses were also obtained if equal doses of SP were given repeatedly.

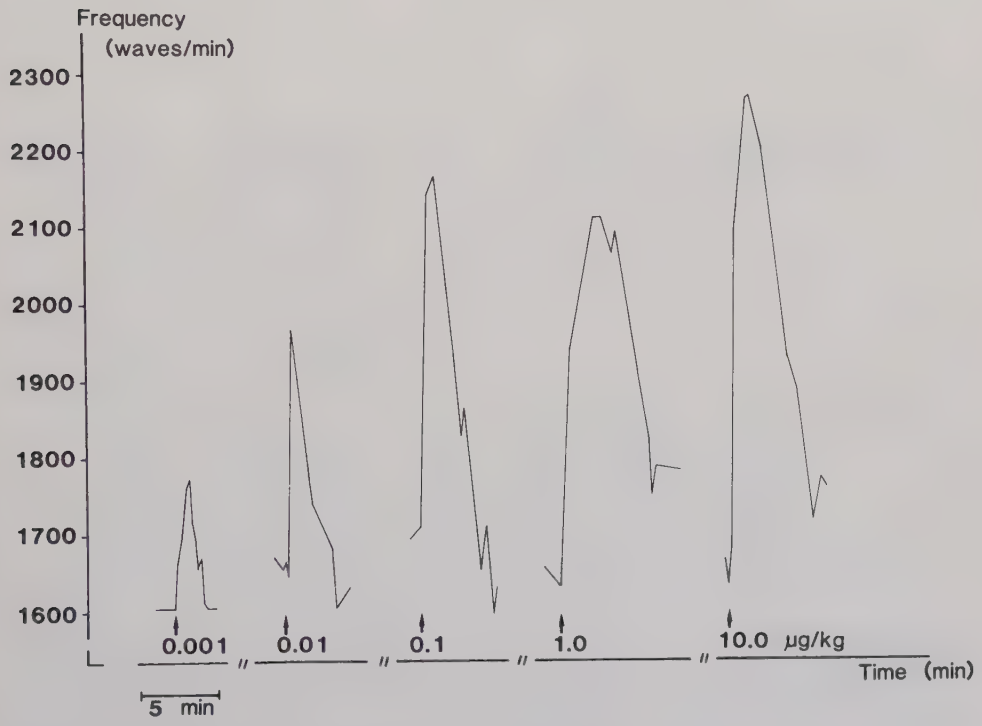

Fig. 5. The effect on the mucociliary wave frequency in 1 rabbit after close intraarterial injections of SP in the dose range 0.001 - 10.0 µg/kg.

TABLE I

The effect of 0.01-10.0 µg/kg SP on mucociliary wave frequency compared with baseline variations and final level of mucocilary activity.

0.01 µg/kg

Rabbit No.	Baseline level (range)	Zero level	Max response	Final level
1	1640 ± 30 (1605-1674)	1656	1968 (18.8%)	1693
2	1436 ± 51 (1380-1480)	1368	1521 (11.2%)	1351
3	1914 ± 87 (1795-2026)	1951	2139 (9.6%)	2037
4	1304 ± 4 (1299-1309)	1344	1506 (12.0%)	1276
5	1386 ± 44 (1353-1458)	1417	1668 (17.7%)	1414
6	1111 ± 24 (1075-1138)	1188	1332 (12.1%)	1174
7	1513 ± 91 (1360-1575)	1411	1650 (16.9%)	1405
		1476 ± 251	14.0 ± 3.6%	1479 ± 294

0.1 µg/kg

Rabbit No.	Baseline level (range)	Zero level	Max response	Final level
1	1700 ± 40 (1644-1735)	1755	2169 (23.6%)	1632
2	1566 ± 83 (1471-1614)	1584	1896 (19.7%)	1570
3	1696 ± 30 (1644-1722)	1624	1878 (15.6%)	1666
4	1587 ± 51 (1531-1647)	1492	1962 (31.5%)	1390
5	1382 ± 34 (1335-1428)	1389	2022 (45.6%)	1389
6	1185 ± 17 (1162-1204)	1155	1527 (32.2%)	1170
7	1343 ± 23 (1320-1375)	1312	1554 (18.5%)	1347
		1473 ± 204	26.7 ± 10.5%	1452 ± 178

1.0 µg/kg

Rabbit No.	Baseline level (range)	Zero level	Max response	Final level
1	1642 ± 15 (1618-1657)	1632	2115 (29.6%)	1743
2	1290 ± 25 (1258-1323)	1351	1668 (23.5%)	1359
3	1803 ± 63 (1717-1887)	1864	2574 (38.1%)	1902
4	1337 ± 56 (1270-1399)	1341	1977 (47.4%)	1389
5	1343 ± 19 (1314-1363)	1341	2178 (62.4%)	1270
6	1112 ± 83 (1029-1227)	1170	1713 (46.4%)	1264
7	1309 ± 72 (1258-1302)	1260	1695 (34.5%)	1312
		1423 ± 241	40.3 ± 13.0%	1463 ± 254

10.0 µg/kg

Rabbit No.	Baseline level (range)	Zero level	Max response	Final level
1	1660 ± 34 (1621-1701)	1665	2271 (36.4%)	1686
2	1509 ± 47 (1432-1561)	1453	2070 (42.5%)	1389
3	1745 ± 32 (1710-1788)	1795	2202 (22.7%)	1671
4	1280 ± 18 (1249-1296)	1254	1848 (47.4%)	1327
5	1308 ± 35 (1269-1338)	1398	2262 (61.8%)	1375
6	1185 ± 61 (1101-1252)	1047	1806 (72.5%)	1053
7	1417 ± 49 (1341-1468)	1405	1965 (39.9%)	1468
		1431 ± 248	46.2 ± 16.5%	1424 ± 217

Footnote. All results are given as means ± S.D. Baseline level refers to the mucociliary wave frequency during 5 min immediately preceding the injection of SP. Zero level is the frequency at the time of injection and final level is the frequency 10 min after the administration of SP. Max response figures in brackets are the percentage increase induced by SP, and used for the statistiscal calculations in the result section. 2-way analysis of variance of the material reveals that there is no difference between zero levels and final levels ($p>0.50$), while the difference in zero levels between individuals is of high statistical significance ($p<0.001$), as well as the effect of different doses of SP ($p<0.001$).

The anticholinergic substance atropine (200 µg/kg) did not affect the SP-induced acceleration or the duration of the response when given 2 min prior to injection of SP. In fact, the dose-response curves were identical as can be seen in Fig. 6 which summarizes the results of 26 experiments in 8 rabbits.

Contralateral injection of SP (16 experiments in 7 rabbits) resulted in a significantly reduced response compared to a corresponding ipsilateral injection in the same animal ($p<0.001$). Moreover, the response was delayed, as illustrated in Fig. 7 which shows the effect of 1.0 µg/kg SP injected ipsilaterally and contralaterally in 6 rabbits.

Adverse reactions were rare. Two of the rabbits receiving 10.0 µg/kg leu-enkephalin showed signs of respiratory depression and 5 of the rabbits given bombesin (10.0 µg/kg) defecated. VIP and SP were well tolerated by the animals and no obvious side-effects were seen.

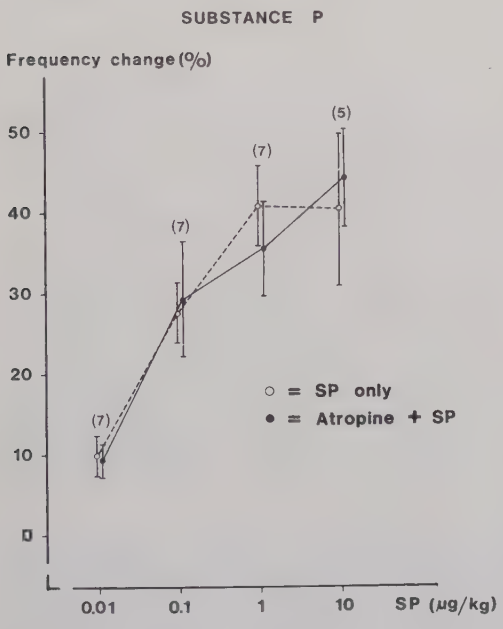

Fig. 6. The dose-response curves after injection of SP intraarterially with and without pretreatment with atropine. Figures in brackets refer to the number of experiments performed. Means and SEM (n=8). Frequency zero level was 1311 ± 262 waves/min (mean ± SD).

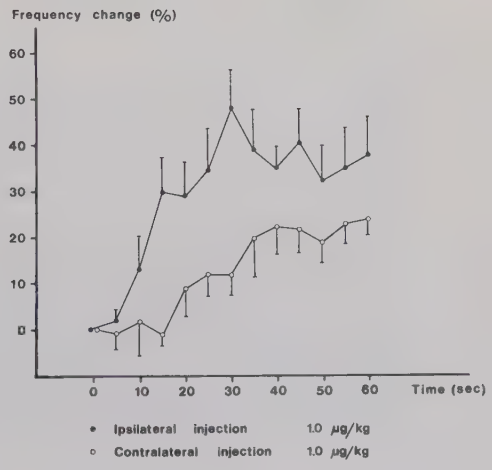

Fig. 7. The results of ipsilateral and contralateral intraarterial injections of 1.0 µg/kg SP. Means and SEM (n=6). Frequency zero level was 1378 ± 260 waves/min (mean ± SD).

Discussion

No reports of the in vivo effects of neuropeptides on mucociliary function have hitherto been published. The findings in the present investigation will therefore be discussed in the light of our knowledge of in vitro or in vivo peptide effects in other organ systems.

In vivo pharmacological and physiological experiments with neuropeptides are hampered by the fact that these compounds are rather unstable and rapidly inactivated in the circulation. Thus it is necessary to administer the investigated peptides as close to the target organ as possibly, and to register their effects immediately and continuously. These requirements are satisfied in the present experimental setup.

In some experimental models VIP has been shown to have secretory effects. Thus, combined infusion of VIP and acetylcholine resulted in a marked potentiation of both vasodilatation and secretion in the submandibular gland in cats (25). On the other hand, infusion of VIP alone provided vasodilatation only, not secretion. VIP relaxes the taenia coli in guinea pigs (26) and is proposed to be a relaxatory neurotransmitter (27). Coles et al 1981 (28) noticed that VIP has an in vitro inhibitory effect on the release of glucoconjugates from secretory cells. The effects of VIP did not, however, influence mucociliary activity in rabbit maxillary sinus in the present study, neither immediately after the intraarterial injection, nor during the ensuing 10-15 min (Fig. 1). Since mucociliary activity is readily accelerated by methacholine (2), and VIP and acetylcholine have been shown to exist in the same neurons (25), there is still the possibility that VIP may potentiate this effect. However, this aspect was not investigated in the present study.

Enkephalin-like immunoreactivity has been found in cell bodies and nerve terminals in the brain and the spinal cord (29). There is substantial evidence suggesting a role for enkephalins in the control of pain, which seems to be their main central action. In the peripheral autonomic nervous system enkephalin--immunoreactive nerve terminals form a dense network around the majority of the ganglion cells of the sympathetic ganglia of rat and guinea pig (30). Enkephalin-like immunoreactivity is also found in the cells of the adrenal medulla and in the preganglionic fibres innervating these cells (31). These findings suggest that enkephalins and catecholamines coexist in the same neurons (9). The effects of adrenergic compounds on the mucociliary activity were investigated by Hybbinette & Mercke (8) using the present test model. β_2-stimulators

like salbutamol accelerated the mucociliary activity. However, their intrinsic activity and potency were smaller than the methacholine-induced effects. Both α_1 and α_2 agonists (phenylephrine and oxymetazoline, respectively) decelerated mucociliary wave frequency. Thus there is pharmacological evidence that the sympathetic part of the autonomic nervous system is able to influence mucociliary activity. No effect of enkephalins on mucociliary activity, corresponding to the above-mentioned properties of adrenergic compounds, was seen in this investigation (Fig. 2).

Bombesin, known to contract smooth muscles in different organs (32), did have a slight effect on mucociliary activity (Fig. 3). The effect was noticed only at 0.1-10.0 µg/kg, and consisted of a small increase in mucociliary wave frequency of at most 14.2%. This weak response makes it very doubtful whether the effect is of any physiological importance.

The discovery that SP accelerates mucociliary activity is interesting. SP is probably a transmitter in unmyelinated primary afferent neurons (33,34,35). SP also occurs in the vagus nerve (36,37), has widespread locations in the central nervous system including interneurons in the spinal cord (38), and thus one might expect it to have both central and peripheral actions. SP has very potent motor action in the periphery. Thus it has potent vasodilating action in the human forearm (39), and it is the neurogenic mediator of antidromic vasodilatation and plasma extravasation (40). In the eye, SP is released upon trigeminal nerve stimulation and causes an inflammatory reaction consisting of miosis and protein leakage in the anterior chamber of the eye, a process which is inhibited by the SP-antagonist (D-Pro^2, D-Trp^{7,9})SP (41,42). In the upper airways, treatment of rats with the SP-releasing drug capsaicin causes increased capillary permeability with leakage of Evans blue in the nasal mucosa. It has therefore been suggested that SP participates in inflammatory reactions in the nasal and paranasal sinuses (43). It has also been shown in <u>in vitro</u> experiments with secretory cells from the airways, that SP has a strong stimulatory effect on mucin production (44).

The SP-induced acceleration of mucociliary activity is remarkably strong, and the effects are similar to those induced by methacholine in the same test system (Figs. 4 and 5). The effect of methacholine, however, is blocked almost completely by atropine 200 µg/kg (2), whereas the same drug has no influence at all on the SP response (Fig. 6).

Addicks et al (45) have given electron microscopical evidence of very intense innervation of the mucosa in the rabbit maxillary sinus, with intraepithelial varicosities in close connection with all types of epithelial cells in the respiratory ciliated epithelium. The varicosities were in the immediate vicinity of epithelial cells, the distance between them corresponding to the synaptic cleft. From a teleological point of view it is tempting to suggest that the respiratory

epithelium is provided with a mucociliary accelerating mechanism, perhaps through an axon reflex releasing endogenous SP. This mechanism would then respond to chemical irritation or to sensory stimulation, e.g. contamination of the mucosa by bacteria or other foreign materials. An interesting aspect has been reported by Wanner et al (46), who described accelerating *in vitro* effects of other inflammatory mediators on mucociliary activity. Ciliary beat frequency was increased 7-33% by prostaglandins E_1 and E_2, as well as leukotriene C_4. This emphasizes the need for research concerning the influence of inflammatory mediators on mucociliary activity.

The mechanism through which SP induces its mucociliary action is probably peripheral. This conclusion is based on the great difference of effects between contralaterally and ipsilaterally injected SP (Fig. 7). The contralaterally induced response comes later, probably due to the injected substance being recirculated via the veins to the ipsilateral side before any effect is possible.

The finding that SP accelerates mucociliary activity raises several questions. A difficult one is whether the acceleration is a result of altered properties of the respiratory tract fluids, or a result of a stimulation of the ciliated cells. It would be interesting to find out whether the observed effect could be blocked by SP antagonists and also if it could be repeated by stimulation of the peripheral trigeminal branch, or produced by SP-releasing drugs.

Acknowledgements

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Substance P antagonists and mucociliary activity in rabbit

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Summary. Substance P (SP) is known to accelerate mucociliary (m.c.) activity in the rabbit maxillary sinus in vivo. The physiological significance of this finding was investigated by testing three putative SP antagonists. [Arg^5, D-Trp^{7,9}, Nle^{11}]SP$_{5-11}$ could not be used as an antagonist because it stimulated m.c. activity. [D-Arg^1, D-Trp^{7,9}, Leu^{11}]SP had no effect on the m.c. activity changes induced by SP. [D-Pro^2, D-Trp^{7,9}]SP was found to be an effective antagonist, 1 mg/kg of this drug reversibly inhibiting both the effects of 0.1 μg/kg SP and the stimulating effect of 1.0 μg/kg bradykinin and 30.0 μg/kg capsaicin; the stimulating effect of 0.5 μg/kg methacholine was not inhibited. It is suggested that bradykinin and capsaicin stimulate m.c. activity at least partly by releasing SP.

The results of this investigation also support the view that the accelerating effect of SP on m.c. activity reflects physiological SP-mediated protective mechanisms in the airways. It is concluded that [D-Pro^2, D-Trp^{7,9}]SP is a useful pharmacological tool for studying the role of SP in the control of m.c. activity in rabbits.

Key words: Mucociliary activity – Substance P – Substance P/antagonists and inhibitors – Maxillary sinus – Capsaicin

Introduction

Several mediators of anaphylaxis and inflammation influence mucociliary (m.c.) activity in the airways. For example, Wanner et al. (1983) investigated the in vitro effects of prostaglandins E1, E2, leukotriene C4 and histamine on sheep tracheal explants and found that all these mediators accelerated ciliary beat frequency. Another mediator involved in inflammatory mechanisms is substance P (SP), the neurogenic mediator responsible for antidromic vasodilation and plasma extravasation (Lembeck and Holzer 1979). SP or closely related peptides are found below and within the nasal epithelium in nerve endings originating in the trigeminal ganglion and also in nerve endings in the sphenopalatine ganglion (Änggård et al. 1979; Lundblad et al. 1983a; Uddman et al. 1983). In the lower airways, the trachea and stem bronchi are rich in SP (Lundberg et al. 1983a). Capsaicin treatment of neonatal rats reduces tissue levels of SP (Gamse et al. 1980) and causes degeneration of chemosensitive sensory afferents (Jancsó et al. 1977), especially unmyelinated C-fibres (Welk et al. 1984). This infers that SP is a transmittor in such fibres, probably with receptors occurring in such parts of the airways as the nasal mucosa, larynx, trachea and main bronchi (Widdicombe 1981). However, it must be emphasized that capsaicin also releases other tachykinins as well (Saria et al. 1985), suggesting that there may be several SP-like transmitters in these fibres.

Using an animal model, the in vivo effect of SP on m.c. activity in the rabbit maxillary sinus has been investigated by Lindberg et al. (1984). SP caused a powerful dose-dependent acceleration of m.c. activity comparable to that obtained with methacholine in the same model (Hybbinette and Mercke 1982a). The effect was resistant to pretreatment with atropine, indicating that the effect is not mediated through cholinergic pathways.

The physiological role of SP in the respiratory tract has been studied by Lundblad and co-workers who showed that SP mediates protective mechanisms in the upper airways, such as protein extravasation (Lundblad et al. 1983b) and vasodilatation (Lundblad et al. 1983c). In the lower airways a similar mechanism is activated by chemical irritation from e.g. formalin, ether and cigarette smoking, inducing plasma extravasation probably through release of SP (Lundberg and Saria 1983). It would be interesting both from a physiological and from a clinical point of view to find out if the acceleration of m.c. activity induced by SP constitutes a similar protective mechanism in the maxillary sinus.

The role of SP in the acceleration of m.c. activity might be elucidated by using SP antagonists. So far, the effect of SP antagonists in rabbit airways has not been described. Several SP antagonists are available, all being analogs of SP or truncated SP with D-amino acid substitutions (see Folkers et al. 1981, 1984; Caranikas et al. 1982).

The aims of the present investigation were therefore: (1) To study if SP antagonists inhibit the effect of SP on m.c. activity in rabbits. (2) To select from three available SP antagonists one which has a distinct and reversible blocking effect. (3) To use this antagonist in order to study the effect on m.c. activity of exogenous SP as well as of SP released from sensory nerve fibres.

Capsaicin was used on account of its known ability to release SP (Gamse et al. 1979). Experiments were also performed with bradykinin, known to stimulate bronchial C-fibres (Kaufman et al. 1980) and subsequently probably cause release of SP (Zhang et al. 1982; Bynke et al. 1983a, 1984). Both drugs are capable of accelerating m.c. activity (Lindberg and Mercke, unpublished results). Methacholine was used to stimulate m.c. activity by a direct action on muscarine receptors.

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Materials and methods

The experiments were performed on male and female rabbits of a white strain (Swedish native breed) weighing 1.8–2.7 kg. Details of anesthetic and surgical techniques have been reported previously (Hybbinette and Mercke 1982b). The animals were anesthetized with urethane 2 g/kg i.m. as an initial dose, and an extra dose of 0.5 g/kg was given i.v. during the operation. The facial or lingual artery was cannulated, and the cannula continuously perfused with physiological saline 2–5 ml/h in order to keep the inlet open. This i.a. route was used for administering the drugs. The mucosa was exposed via a trepanation hole of about 2 × 8 mm, which was immediately sealed with an antimist window and bone wax (Ethiocon). The m.c. activity was first observed through a binocular microscope. The criterion for a working preparation was properly functioning transportation of particles like air bubbles and shed cells. One of the eyepieces was replaced by a phototransducer and recordings of the m.c. activity were made from the frontosuperior part of the maxillary sinus using a photoelectric technique (Mercke et al. 1974). The m.c. wave pattern was monitored continuously on an oscilloscope and recorded on an ink writer. The recordings were analysed both manually and with the aid of a computerized frequency calculator. The m.c. wave frequency was expressed in waves/min. The induced frequency changes were expressed as percentages of the m.c. wave frequency immediately preceding the administration of the test substances (= frequency zero level). These frequency changes were then used for statistical calculations. The m.c. wave frequency was calculated automatically every 10 s during 5 min after each injection of a test substance and at intervals of 1–5 min otherwise. ECG and rectal temperature were monitored, and body temperature maintained at 37.0–38.5° C with the aid of a heating pad.

The following drugs were tested: SP, bradykinin (Beckman Bioproducts, Geneva, Switzerland), capsaicin, methacholine (Sigma, St. Louis, MO, USA), [D-Pro^2, D-Trp^{7,9}]SP and [Arg^5, D-Trp^{7,9}, Nle^{11}]SP$_{5-11}$ (Ferring, Kiel, FRG) and [D-Arg^1, D-Trp^{7,9}, Leu^{11}]SP (Spantide) (Ferring, Malmö, Sweden). Capsaicin was initially dissolved in saline containing 10% Tween 80 and 10% ethanol, further dilutions were made in saline. Methacholine was dissolved in saline. All the other drugs were dissolved in saline containing 0.5% albumin (Kabi, Stockholm, Sweden) in order to prevent adsorption to glass and plastic surfaces.

Experimental procedure. Initially the m.c. activity was recorded every 1 min until the frequency had been stable for approximately 30 min. The mean value of the frequency variations during the 5 min preceding an injection (= baseline level) was calculated in order to allow comparison both with the frequency zero level, and the final level 10 min after administration of a drug. Agreement of these levels would indicate that the preparation remained unchanged between the different challenges. The test solutions were delivered as bolus doses of 0.2 ml during 3 s through the cannula, except the 1 mg/kg dose of [D-Pro^2, D-Trp^{7,9}]SP and [D-Arg^1, D-Trp^{7,9},Leu^{11}]SP which was given as an arterial infusion of 1 mg/ml for 4 min. Saline controls with and without albumin were used to reveal non-specific effects, i.e. peptides remaining in the arterial cannula which might influence the ensuing experiments. Saline controls were also compared with the effects of SP, capsaicin and bradykinin after pharmacological blockade.

The following series of experiments were performed:

(1) The three antagonists were first tested for their ability to stimulate m.c. activity; log dose-response curves for the dose range 0.1–100 µg/kg were plotted. For two of the antagonists a dose of 1,000 µg/kg was also tested. The various doses were added consecutively. The interval between administrations was approximately 20 min, in most cases the lowest dose (0.1 µg/kg) was tested first.

(2) SP 0.1 µg/kg was given 4–10 min after the injection of 100 µg/kg of the antagonist to be tested, and the results compared with the increase of m.c. activity induced by the same dose of SP in the same rabbit but without pretreatment with the blocker.

(3) In order to estimate the duration of pharmacological blockade control experiments were first made with repeated doses of 0.1 µg/kg SP given at intervals of about 5 min (exact intervals are seen in Fig. 2). The same animals were then pretreated with one of the two SP antagonists [D-Pro^2, D-Trp^{7,9}]SP or [D-Arg^1, D-Trp^{7,9}, Leu^{11}]SP 1 mg/kg, and the same sequence of doses of SP was given again. Two hours later the experimental series was repeated after pretreatment with the other antagonist.

(4) [D-Pro^2, D-Trp^{7,9}] SP 1 mg/kg was given 10 min prior to the injection of 0.1 µg/kg SP. The results were compared to controls with 0.1 µg/kg SP in the same animal at least 1 h after the administration of the antagonist.

(5) Bradykinin 1.0 µg/kg and capsaicin 30.0 µg/kg were given 20 min after [D-Pro^2, D-Trp^{7,9}]SP 1 mg/kg. The same animals were then challeged with a repeated dose of bradykinin 1.0 µg/kg or capsaicin 30.0 µg/kg 2 h later, and the effects on m.c. activity were compared (control experiments).

(6) Methacholine 0.5 µg/kg was given 10 or 20 min after [D-Pro^2, D-Trp^{7,9}]SP 1 mg/kg in order to study any non-specific inhibition of the antagonist on cholinergically stimulated m.c. activity. Methacholine was also given to the same animals either 1–2 h later or 30 min preceding the administration of the SP antagonist and the effects were compared.

Each rabbit served as its own control. In order to avoid the bias of a possible time-dependent deterioration in the experimental set-up producing a false blockade during the experiments with [D-Pro^2, D-Trp^{7,9}]SP 1 mg/kg, all controls were run after the effect of the SP antagonist had disappeared. The results from these control experiments were comparable with those obtained in untreated animals which gave stimulatory effects on m.c. activity of SP 0.1 µg/kg ($26.7 \pm 4.0\%$, $n = 7$), bradykinin 1.0 µg/kg ($28.9 \pm 2.6\%$, $n = 10$) and capsaicin 30.0 µg/kg ($36.8 \pm 4.2\%$, $n = 7$).

The results are expressed as means and standard error of the mean (SEM), if not otherwise stated. The statistical analyses were performed by the use of the one-way analysis of variance (dose-response curves), and the two-way analysis of variance (blocking experiments). P-values < 0.05 were considered significant.

Results

[D-Pro^2, D-Trp^{7,9}]SP and [D-Arg^1, D-Trp^{7,9}, Leu^{11}]SP were without significant effect on m.c. activity in the dose range 0.1–1,000 µg/kg. In contrast [Arg^5, D-Trp^{7,9}, Nle^{11}]SP$_{5-11}$ induced a strong stimulation in the dose range 0.1–100 µg/kg, the maximal effect being $28.2 \pm 5.5\%$ ($P < 0.05$ for the

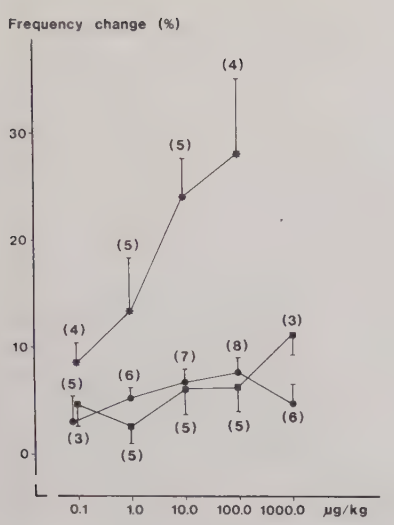

Fig. 1. Log dose-response curves showing the stimulating effects of [Arg^5, D-Trp^{7,9}, Nle^{11}]SP$_{5-11}$ (★——★) in 5 rabbits compared to the non-significant actions of [D-Pro^2, D-Trp^{7,9}]SP (●——●) in 9 rabbits and [D-Arg^1, D-Trp^{7,9}, Leu^{11}]SP (■——■) in 5 rabbits. Figures in brackets refer to number of experiments performed at each dose. The results are expressed as means and SEM. Mean frequency zero level was 1,374 ± 214 waves/min (mean ± SD)

Table 1. The effect of three putative SP antagonists (100 µg/kg) on m.c. stimulation induced by 0.1 µg/kg SP

Antagonist	n	Antagonist + SP	Control (SP)
[D-Pro^2, D-Trp^{7,9}]SP	9	20.5 ± 4.0	27.2 ± 2.8 $P < 0.01$
[Arg^5, D-Trp^{7,9}, Nle^{11}]SP$_{5-11}$	4	25.4 ± 3.1	31.7 ± 4.6 NS ($P = 0.14$)
[D-Arg^1, D-Trp^{7,9}, Leu^{11}]SP	5	22.7 ± 4.3	28.7 ± 5.7 NS ($P = 0.09$)

Values are frequency changes expressed as percentages of the m.c. wave frequency immediately preceding the administration of 0.1 µg/kg SP. Values are means and SEM. Statistical evaluation by the two-way analysis of variance

whole curve) (Fig. 1). The stimulation could be repeatedly evoked by renewed administration of the drug.

Of the three SP analogs tested at 100 µg/kg, only [D-Pro^2, D-Trp^{7,9}]SP had a significant antagonistic effect when given 4–10 min prior to the injection of 0.1 µg/kg SP, as summarized in Table 1.

The effect of 0.1 µg/kg SP on the m.c. activity in the rabbit maxillary sinus was reproducible, without any overt tachyphylaxis. Figure 2 shows the results of injection of SP and [D-Pro^2, D-Trp^{7,9}]SP in one of the two rabbits consecutively pretreated with [D-Pro^2, D-Trp^{7,9}]SP and [D-Arg^1, D-Trp^{7,9}, Leu^{11}]SP. Repeated injections of 0.1 µg/kg SP after pretreatment with 1 mg/kg [D-Pro^2, D-Trp^{7,9}]SP revealed a blockade of the SP receptors during the first 30 min, with maximum blockade occurring 10–20 min after cessation of the arterial infusion (Fig. 2). On the other hand [D-Arg^1, D-Trp^{7,9}, Leu^{11}]SP 1 mg/kg had no inhibiting effect. Mucociliary activity was accelerated 26.0 ± 3.8% by 0.1 µg/kg SP after pretreatment with this compound against 26.8 ± 2.3% without pretreatment ($n = 3$).

[D-Pro^2, D-Trp^{7,9}]SP was then subjected to further evaluation. The effect of 0.1 µg/kg SP was reduced by the antagonist as compared to controls with SP given to the same animal more than 1 h later (range 76–93 min). The resulting acceleration with the SP antagonist was 7.8 ± 1.0% and without 32.3 ± 3.4% ($n = 6$, $P < 0.01$). The time course of these experiments is shown in Fig. 3. The effect of 0.1 µg/kg SP was apparent after 10–20 s and the peak value was reached after about 1 min. It may also be noted that the effect of 0.1 µg/kg SP was not entirely inhibited by pretreatment with the antagonist, although the slight persisting effect was delayed. Stimulating effects of 1 mg/kg [D-Pro^2, D-Trp^{7,9}]SP were not seen, the increase in m.c. activity being 5.3 ± 1.4% for saline controls and 4.2 ± 1.9% for the antagonist ($n = 6$).

Pretreatment with 1 mg/kg [D-Pro^2, D-Trp^{7,9}]SP reduced the effects of 1.0 µg/kg bradykinin ($n = 3$) and of 30.0 µg/mg capsaicin ($n = 3$) on m.c. activity as illustrated in Fig. 4. The effect of bradykinin diminished from 26.3 ± 8.6% to 6.7 ± 1.0%, while the effect of capsaicin was reduced from 33.0 ± 3.1% to 10.7 ± 3.9%. There was no difference between the results of the control experiments, run after the effect of the SP antagonist had disappeared, and results obtained in untreated animals.

Methacholine 0.5 µg/kg stimulated m.c. activity irrespective of SP-receptor blockade by 1 mg/kg [D-Pro^2, D-Trp^{7,9}]SP. The acceleration was 25.4 ± 1.5% during blockade and 28.0 ± 2.2% otherwise ($n = 6$, $P = 0.22$).

Discussion

Of the three putative SP antagonists tested, only [D-Pro^2, D-Trp^{7,9}]SP displayed distinct inhibition of the acceleration of m.c. activity induced by SP in the rabbit maxillary sinus (see Table 1). However, one cannot be entirely sure that the other two compounds do not function similarly as well. One essential criterion that an SP antagonist has to meet as a pharmacological tool is a minimum of stimulating effects per se. Obviously [Arg^5, D-Trp^{7,9}, Nle^{11}]SP$_{5-11}$ did not fulfil this criterion (see Fig. 1), and this blocker was therefore abandoned in the subsequent experiments.

In the present study, injection of methacholine 20 min after administering the antagonist still produced a prompt acceleration of m.c. activity, indicating that the antagonist did not interfere with responses mediated through muscarine receptors. The antagonistic effect was reversible, and it was possible to run all controls in the same animals either before, or at least 1 h after the administration of the antagonist.

Basic peptides, like SP (Johnson and Erdös 1973), and several SP antagonists (Håkanson et al. 1983; Folkers et al. 1984) are known to release histamine from rat peritoneal mast cells. This effect probably does not play any role in the present experiments, since histamine is a fairly weak stimulator of m.c. activity (Wanner et al. 1983), and high doses of histamine are required before any in vivo effect is noticeable (Lindberg and Mercke, unpublished results). In any case, [D-Pro^2, D-Trp^{7,9}]SP did not exhibit any significant stimulating effect.

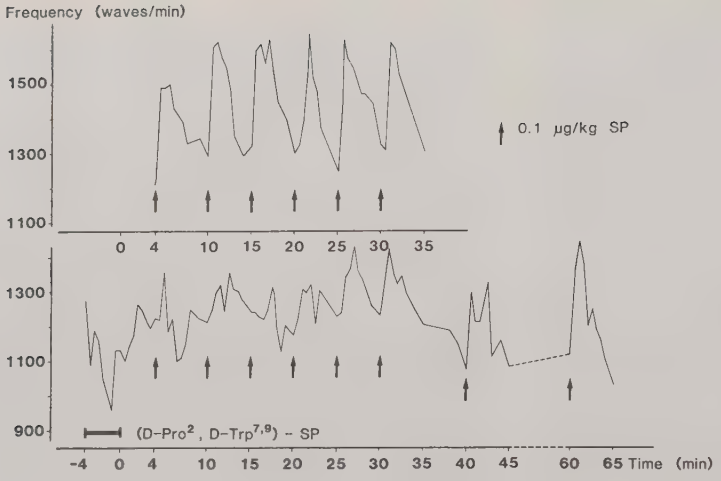

Fig. 2
Effect on m.c. wave frequency in one rabbit given repeated injections of 0.1 µg/kg SP (*above*), and 2 h later after pretreatment with 1 mg/kg [D-Pro², D-Trp⁷,⁹]SP given as a 4 min arterial infusion (*below*). The effect of the antagonist diminishes after 30 min, and disappears completely after 1 h

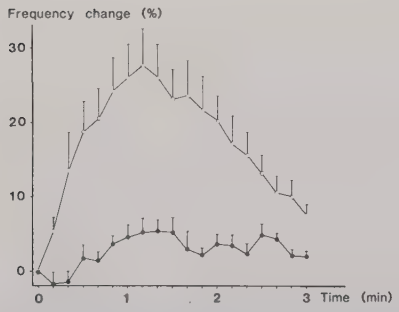

Fig. 3. The time course for the effect of a single dose of 0.1 µg/kg SP after pretreatment with 1 mg/kg [D-Pro², D-Trp⁷,⁹]SP (●——●), compared with controls in the same animals (○——○). The injections were made at zero time. The results are expressed as means and SEM. Frequency zero level was $1,282 \pm 201$ waves/min (mean $\pm$ SD) ($n = 6$, $P < 0.01$)

[D-Arg¹, D-Trp⁷,⁹, Leu¹¹]SP (Spantide) was found to be inactive as an antagonist at a dose of 1 mg/kg. This is somewhat surprising because in experiments on isolated preparations of guinea-pig ileum (Rosell et al. 1983) or guinea-pig taenia coli (Folkers et al. 1984), [D-Arg¹, D-Trp⁷,⁹, Leu¹¹]SP was found to be a more potent antagonist than [D-Pro², D-Trp⁷,⁹]SP. However, in the rabbit eye the in vivo effects of the two antagonists were almost identical (Bynke 1984), indicating that differences between organs and species may exist. Another possible explanation is that [D-Pro², D-Trp⁷,⁹]SP may have a longer in vivo half-life or better bioavailability than [D-Arg¹, Trp⁷,⁹, Leu¹¹]SP.

The SP antagonists are thought to be competitive antagonists, as indicated by in vitro experiments using guinea-pig ileum or taenia coli (Håkanson et al. 1982; Rosell et al. 1983; Folkers et al. 1984). Although in the present investigation the antagonistic effect of [D-Pro², D-Trp⁷,⁹]SP at 1 mg/kg was unmistakable, it should be noted that the concentration of the antagonist had to be 10^4 times larger than that of SP, before blockade could be demonstrated. Moreover, it is clear that even at this high dose there was no complete inhibition since a total blockade would have resulted in a curve closer to the zero level on the y-axis (see Fig. 3).

Hökfelt et al. (1981) reported that the SP antagonist [D-Pro², D-Trp⁷,⁹]SP caused local morphological changes after injection into the ventral mesencephalon in rats. Gallacher (1983), when studying rat parotid gland function in vitro, observed that prolonged exposure (10 – 20 min) to [D-Pro², D-Trp⁷,⁹]SP induced irreversible functional changes. This effect of [D-Pro², D-Trp⁷,⁹]SP on parotid gland function was time-dependent, prolonged exposure to the blocker being required (more than 6 – 8 min). However, no such functional changes of m.c. activity attributable to [D-Pro², D-Trp⁷,⁹]SP were seen in the present investigation. Also, the effect of [D-Pro², D-Trp⁷,⁹]SP was clearly reversible (see Fig. 2). On the other hand, a functional change in the C-fibres, influencing the release of SP induced by capsaicin or bradykinin could not be excluded, although it seems less probable.

Relatively few in vivo studies have been performed with SP-antagonists. In the rabbit eye [D-Pro², D-Trp⁷,⁹]SP has been shown to inhibit not only the effects of SP on the pupillary diameter and on the blood-aqueous barrier in the anterior chamber but also the effects of bradykinin and capsaicin (Bynke et al. 1983a, b; 1984). It was suggested that the effects of bradykinin and capsaicin were mediated by the release of SP. In the present investigation the accelerating effect on m.c. activity of both drugs was reversibly diminished by the SP antagonist (Fig. 4), indicating that the influence on m.c. activity of these drugs, at least in part may be explained by release of SP.

The in vivo effect of SP antagonists in the airways has also been investigated by Lundberg and co-workers (Lundberg et al. 1984). They found that cigarette smoke caused a local oedema in the nasal mucosa in rats, as indicated by extravasation of Evans blue. This oedema was

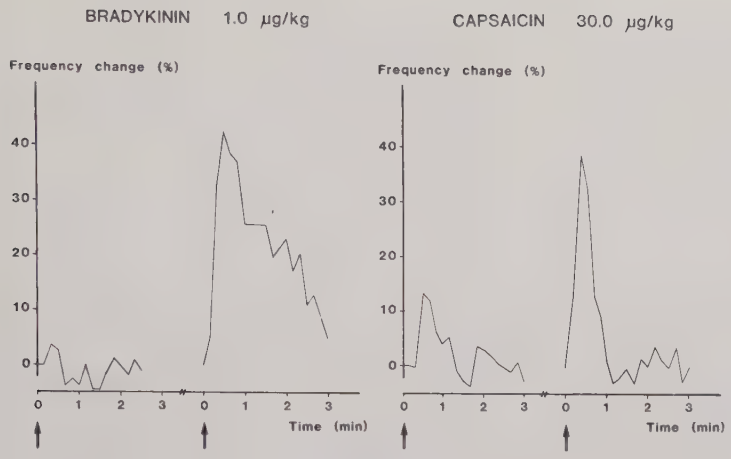

Fig. 4
[D-Pro^2, D-Trp^{7,9}]SP 1 mg/kg inhibits the m.c. response of 1.0 µg/kg bradykinin and 30.0 µg/kg capsaicin. Results from one rabbit are shown for each substance, antagonistic effect is shown to the left and controls to the right

abolished or significantly reduced by local or systemic pretreatment by [D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11}]SP, an SP antagonist not investigated in the present study. The same antagonist also inhibited the increase in insufflation pressure caused by vagal stimulation in guinea pigs (Lundberg et al. 1983b). Together these findings suggest that nerve fibres containing SP may exert local control of vascular permeability and smooth muscle tone in the airways. Also, the finding that SP accelerates m.c. activity, suggests that endogenous SP, or an SP-like tachykinin, is involved in protective mechanisms in the airways. Further research regarding this aspect should be facilitated by the results of the present investigation, showing [D-Pro^2, D-Trp^{7,9}]SP to be an inhibitor of the effect of SP on m.c. activity in rabbits.

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Bradykinin Accelerates Mucociliary Activity in Rabbit Maxillary Sinus

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Lindberg S, Mercke U. Bradykinin accelerates mucociliary activity in rabbit maxillary sinus. Acta Otolaryngol (Stockh).

The in vivo effect of bradykinin on mucociliary (m.c.) activity in the rabbit maxillary sinus was investigated by administering the substance (0.01–10.0 μg/kg) via arteria maxillaris and recording the responses with a non-invasive photoelectric technique. Bradykinin accelerated the m.c. activity in a dose-dependent manner in the dose range 0.05–10.0 μg/kg. While the action of bradykinin was resistant to pretreatment with indomethacin 10.0 mg/kg, it was considerably weakened by the substance P antagonist [D-Pro^2,D-Trp^{7,9}]SP 1 mg/kg. The initial effect of bradykinin was also suppressed by atropine, suggesting that bradykinin accelerates m.c. activity by a dual mechanism comprising both SP and acetylcholine. Bradykinin probably stimulates a reflex arc in the airways involving afferent SP neurons and efferent post-ganglionic parasympathetic neurons. *Key words: mucociliary activity, bradykinin, substance P, indomethacin, atropine, substance P/antagonists & inhibitors, maxillary sinus.*

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The mucociliary (m.c.) system is an essential defence mechanism in the airways, the removal of bacteria, cellular debris and other materials being particularly important in diseases in the respiratory epithelium. Since an increase in m.c. activity seems beneficial during inflammatory reactions of the airways, an autonomous accelerating mechanism might be expected. Indirect evidence of such a mechanism is given by Ahmed et al. (1) who studied the effect of the drug FPL-55712—a slow-reacting substance of anaphylaxis (SRS-A) antagonist—on 6 asymptomatic non-smokers with ragweed asthma. They found that while tracheal mucus transport slowed down by ragweed extract challenge, pretreatment with FPL-55712 instead accelerated the mucus transport above the initial level during the provocations. They concluded that SRS-A impaired m.c. velocity, while other mediators of anaphylaxis may have a stimulatory effect.

The neuropeptide substance P (SP) is one probable accelerator of m.c. activity during inflammation (2). SP has been put forward as a mediator of neurogenic inflammation in general (3). SP occurs in nerve fibres terminating in the subepithelial layer in the mucosa of the upper airways (4, 5, 6). The SP nerve fibres probably originate in the trigeminal ganglion. Sometimes the SP-immunoreactive nerve endings are seen to penetrate into the epithelium. Intraarterial injection of SP accelerates m.c. activity in the rabbit maxillary sinus (2). The effect is dose-dependent and comparable to the accelerating effect of the cholinergic agonist methacholine on the same preparation (7). However, the effect of SP is resistant to atropine, implying that the effect does not depend upon cholinergic nerves.

Another basic peptide thought to be involved in inflammatory reactions is the nonapeptide bradykinin. The occurrence of bradykinin in nasal secretions was proposed by Eccles & Wilson (8). The effect of bradykinin on m.c. activity is not, however, known.

The role of bradykinin and SP on inflammatory reactions has been studied in the rabbit eye. Both peptides evoke miosis and ocular hypertension after intracameral administra-

tion. However, the effect of bradykinin was dependent on an intact fifth cranial nerve (9, 10), and was blocked by the SP antagonists [D-Pro^2,D-Trp^{7,9}]SP and [Arg^5,D-Trp^{7,9}]SP$_{5-11}$ (11). These findings suggest that bradykinin at least partly causes inflammation in the rabbit eye by release of SP. If bradykinin stimulates m.c. activity, this could be mediated as a consequence of the release of SP.

However, bradykinin is also a potent stimulator of prostaglandin synthesis, and some prostaglandins, i.e. PGE_1 (12, 13) and PGE_2 (13), accelerate m.c. activity in vitro. It is therefore necessary to distinguish the role of SP and the role of prostaglandins on the m.c. accelerating mechanisms when studying the effect of bradykinin. This could be done by using the pharmacological antagonists [D-Pro^2,D-Trp^{7,9}]SP and indomethacin. [D-Pro^2,D-Trp^{7,9}]SP inhibits the SP-induced effect on m.c. activity (14), while indomethacin is a potent blocker of prostaglandin synthesis (15).

The aim of the present investigation was to study:

1. The in vivo effect of bradykinin on m.c. activity.
2. The mode of action of bradykinin by using the following pharmacological blockades:
   a. Cholinergic blockade of muscarinic receptors with atropine.
   b. Inhibition of prostaglandin synthesis with indomethacin.
   c. Inhibition of SP receptors by the SP antagonist [D-Pro^2,D-Trp^{7,9}]SP.

MATERIALS AND METHODS

The experiments were performed on 40 rabbits of a white strain, raised under controlled conditions and weighing 1.8–3.0 kg (37 males, 3 females). Details of anesthetic and surgical techniques have been described previously (16). The animals were anesthetized with urethane i.m. 2 g/kg as an initial dose, and an extra dose of 0.5 g/kg was given in an ear vein during the operation. The facial artery or lingual artery was cannulated, and the cannula was continuously perfused with physiological saline 2–5 ml/h in order to keep the inlet open. This i.a. route was used for administering the test substances. The mucosa in the fronto-superior part of the maxillary sinus was exposed through a trepanation hole of about 2×8 mm which was immediately sealed with an antimist window and bone wax (Ethicon).

The m.c. activity was observed through this window with a binocular microscope and the transportation of small particles like air bubbles and shed cells was a necessary criterion for a working preparation. One eye-piece was then switched to a phototransducer and the m.c. activity recorded by a photoelectric technique (17). The m.c. wave pattern was monitored continuously on an oscilloscope and recorded on an ink writer. The recordings were analysed by a computerized frequency calculator which expressed the m.c. wave frequency in waves/min. The induced frequency changes were expressed as percentages of the basal m.c. wave frequency (frequency zero level) immediately preceding the administration of the test substances. The m.c. wave frequency was calculated every 10 sec during the experiments, and at intervals of 1–5 min otherwise. ECG and rectal temperature were monitored, and body temperature was adjusted to normal (i.e. 37.0–38.5°C) with the aid of a heating pad.

Bradykinin (Beckman Bioproducts, Geneva, Switzerland) and [D-Pro^2,D-Trp^{7,9}]SP (Ferring, Malmö, Sweden) were dissolved in physiological saline containing 0.5% albumin (Kabi, Sweden) in order to avoid adherence to the walls of glass and plastic cannulas. Atropine sulphate (ACO, Sweden) was dissolved in saline, while indomethacin (Dumex, Denmark) was dissolved in sterile water.

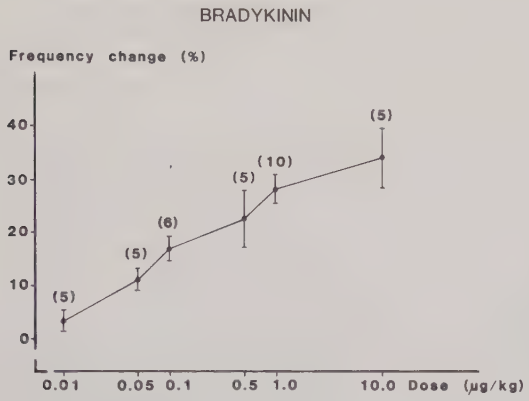

Fig. 1. Bradykinin i.a. dose-dependently accelerates m.c. activity. Figures in brackets refer to number of experiments performed. The results are expressed as means and SEM (standard error of the mean), $n=19$. The correlation coefficient for raw data: $r=0.77$. The frequency zero level was 1 386±140 waves/min.

Experimental procedure

The m.c. activity was recorded once a minute until the frequency had been stable for approximately 30 min. Bradykinin and atropine were delivered as bolus injections of 0.2 ml during 3 sec. Injections of saline with and without albumin served as control experiments and to disclose any traces of peptides remaining in the arterial inlet.

1. The effect of increasing doses of bradykinin was investigated, and a log dose-response curve was plotted in the interval 0.01–10.0 µg/kg (corresponding to 9.4 picomol-9.4 nanomol/kg). Each rabbit was only tested with 2–3 doses of bradykinin, and the time lapse was at least 30 min between injections.
2. Atropine 0.2 mg/kg was given i.a. 2 min before the injection of bradykinin.
3. Indomethacin 10 mg/kg (5 mg/ml) was given in an ear vein 30 min before the injection of bradykinin.
4. The SP antagonist [D-Pro^2,D-Trp^{7,9}]SP 1 mg/kg was given as an arterial infusion (1 mg/ml) during 4 min. The experiments with bradykinin were performed 20 min later. The same animals were challenged with a repeated dose of bradykinin more than two hours later.

The results are expressed as means and SEM (standard error of the mean), unless otherwise indicated. Statistical evaluations were performed by analysis of variance, one-way or two-way. $p<0.05$ was considered significant.

RESULTS

Bradykinin caused a dose-dependent acceleration of m.c. wave frequency in the rabbit maxillary sinus in the dose-range 0.01–10.0 µg/kg. The effect was significant compared to saline for 0.05–10.0 µg/kg of bradykinin. A summary of the results is given in Fig. 1 ($p<0.001$ for the whole curve, one-way analysis of variance).

The bradykinin-produced acceleration of m.c. activity had a latency of 10–20 sec, and a peak within one min, the activity then declined during the 5–10 min that followed. The time course of the effect of 1.0 µg/kg bradykinin is illustrated in Fig. 2*a*, showing that the m.c. activity still remained elevated over the basal level after 5 min. The higher the dose, the longer was the duration of the response. A second dose of bradykinin generally produced a weaker response. For example, 1.0 µg/kg bradykinin injected twice, with an interval of

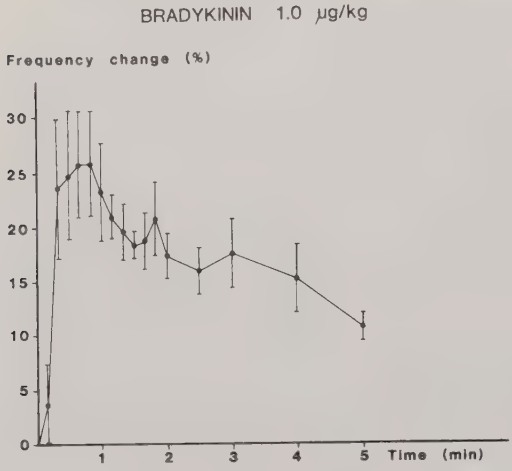

Fig. 2a. Time course of the bradykinin-induced (1.0 µg/kg) acceleration of m.c. activity. The results are expressed as means and SEM (n=7). Frequency zero level was 1272±208 (mean ± SD).

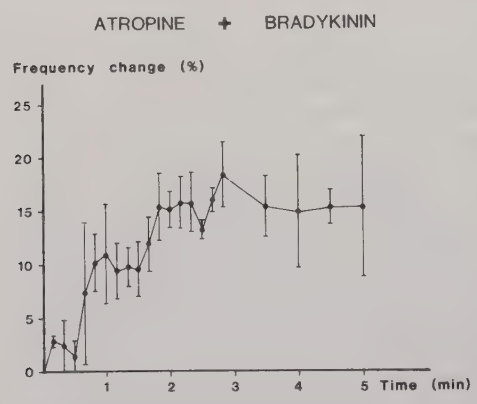

Fig. 2b. Time course of the bradykinin-induced (1.0 µg/kg) acceleration of m.c. activity after pretreatment with 0.2 mg/kg atropine. The results are expressed as means and SEM (n=5). Frequency zero level was 1219±125 (mean ± SD).

more than 30 min between injections, induced an increase of 26.4±2.1% (first injection) compared to 18.7±1.6% (second injection, n=5, p<0.05, two-way analysis of variance).

The blocking experiments summarized in Table I were performed with a dose of 1.0 µg/kg bradykinin. Neither atropine nor indomethacin influenced the peak response to bradykinin. However, it was obvious that atropine prolonged the latency of the response (Fig. 2*b*), thus influencing the initial effect of bradykinin on m.c. activity. This was evident 30 s after injection (Table I). Indomethacin alone was without effect on m.c. activity. Moreover, it did not influence the response to bradykinin (see Table I).

The SP-antagonist [D-Pro^2,D-Trp^{7,9}]SP inhibited the effect of bradykinin (Fig. 3, Table I). The increase was diminished from 23.5±3.0% to 5.3±1.9% (p<0.01, n=7, two-way analysis of variance), which was equal to results of saline controls in the same animals (3.7±1.3%).

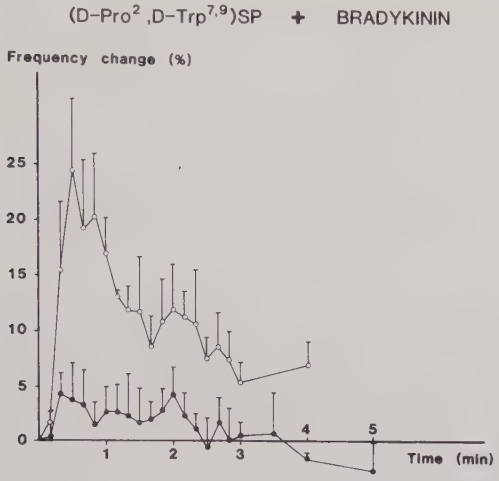

Fig. 3. Time course of the bradykinin-induced acceleration of m.c. activity after pretreatment with 1 mg/kg [D-Pro^2,D-Trp^{7,9}]SP. The upper curve represents the control experiments (bradykinin alone) in the same animals. The results are expressed as means and SEM (n=4). Frequency zero level was 1 478±197 (mean ± SD).

Adverse reactions were seen when the animals were given bradykinin 5.0 and 10.0 μg/kg. Bradycardia, tachypnea and in two cases pain reactions were noticed.

DISCUSSION

The results of the present investigation show that bradykinin accelerates m.c. activity. The acceleration was blocked by the SP antagonist [D-Pro^2,D-Trp^{7,9}]SP 1 mg/kg, suggesting that bradykinin acts through release of endogenous SP, a potent accelerator of m.c.

Table I. *Effect of various antagonists on the acceleration of m.c. activity induced by bradykinin 1.0 μg/kg*

	n	Frequency change (%)	*p*
Peak response			
Bradykinin alone	10	28.9±2.6	
Atropine 0.2 mg/kg + bradykinin	7	23.7±0.9	0.20 NS
Indomethacin 10 mg/kg + bradykinin	7	23.0±1.5	0.16 NS
[D-Pro^2,D-Trp^{7,9}]SP 1 mg/kg + bradykinin	7	5.3±1.9	<0.01
Initial response (30 sec after injection)			
Bradykinin alone	10	20.3±5.4	
Atropine 0.2 mg/kg + bradykinin	7	5.5±1.8	<0.05
Indomethacin 10 mg/kg + bradykinin	7	15.5±4.1	>0.50 NS
[D-Pro^2,D-Trp^{7,9}]SP 1 mg/kg + bradykinin	7	0.7±2.3	<0.01

Statistical evaluations by the one-way analysis of variance, except for the experiments with [D-Pro^2,D-Trp^{7,9}]SP, where two-way analysis of variance was more appropriate.

activity (2). In earlier experiments the same dose of the SP antagonist was shown to reduce the effect of 0.1 μg/kg SP on m.c. activity (14). The present findings are in agreement with the results of experiments with bradykinin and SP in the rabbit eye (9, 11).

An alternative approach to the hypothesis that bradykinin acts by releasing SP was used by Lundberg & Saria (18), who investigated the extravasation of Evans Blue as a parameter of increased vascular permeability in rat tracheal mucosa. SP and bradykinin increased the extravasation. Pretreatment with capsaicin (50 mg/kg s.c. on day 2 of life) did not affect the response to SP whereas the response to bradykinin was greatly reduced. Neonatal pretreatment with capsaicin is known to deplete SP-like immunoreactivity (19, 20) in the nasal mucosa (6), and also to cause degeneration of C-fibre afferents (21).

However, bradykinin also has effects unrelated to SP (22). Thus, the influence of bradykinin on m.c. activity may be direct, or via some other mediator.

The half-life of bradykinin in blood is very short (about 15 sec) (23) and 80–95% of the biological activity of bradykinin is eliminated during the few seconds required for passing through the pulmonary vascular bed (24). Therefore, the rather long duration of the bradykinin-produced effect (see Fig. 2*a*) is probably not due to recirculation of bradykinin, but to the release of other mediators, such as SP or prostaglandins. Unlike SP, bradykinin releases prostaglandins from various organ systems including the lung (25).

The effect of prostaglandins PGE_1 and PGE_2 on m.c. activity was investigated on sheep tracheal explants by Wanner et al. (13). The ciliary beat frequency was increased by 7–33%. An in vivo effect of prostaglandins on m.c. activity is supported by the finding of Richardson et al. (26) that prostaglandins PGA_2, PGE_1, PGE_2, $PGF_{1\alpha}$ and $PGF_{2\alpha}$ all gave an in vivo increase in mucin output in cat trachea.

However, the prostaglandin synthesis inhibitor indomethacin neither influenced the peak response to bradykinin nor the duration of the response. Thus, the stimulation of prostaglandin synthesis induced by bradykinin seems to be of less importance than the SP-releasing effect of the same drug with respect to acceleration of m.c. activity.

Conflicting reports on the effect of bradykinin itself on mucus secretion have appeared. Studies of canine tracheal explants have failed to show any effect on production and release of macromolecules associated with mucus production, whereas SP caused an increase (27). On the other hand, Davis et al. (28), found that both bradykinin and capsaicin caused a marked in vivo increase in the number of hillocks in the canine trachea (elevations due to secretion from the ducts of submucosal glands). Since bradykinin stimulates bronchial C-fibres, as shown by electrophysiological experiments (29), C-fibre activation could mediate the secretory effects of bradykinin. This hypothesis is supported by the results of the present investigation. The concept that bradykinin acts by activating C-fibres in the airways might explain the different effects on secretion in vivo and in vitro.

Although the muscarinic antagonist atropine did not influence either the peak response or the duration of the bradykinin-evoked acceleration, it was obvious that the initial effect was suppressed and the latency prolonged (compare Fig. 2*a* and Fig. 2*b*). This finding suggests that activity in cholinergic (parasympathetic) neurons could play an essential role in prompt acceleration of m.c. activity. In their study Davis et al. (29) found that the effect of bradykinin on submucosal glands in dog trachea was sensitive to atropine, and they concluded that bradykinin stimulated a reflex in the airway via afferent unmyelinated C-fibres and efferent parasympathetic cholinergic neurons. A similar reflex might explain the effect of atropine in the present investigation. This is the more likely, since the accelerating effect on m.c. activity of capsaicin, which also activates C-fibres and releases SP, is influenced by atropine in an identical manner (Lindberg & Mercke, unpublished results). On the other hand the SP antagonist inhibits the effects of both bradykinin and capsaicin (14), suggesting that the cholinergic part of the response is secondary to the release of SP

from the afferent C-fibres. Interestingly, neither the latency of the response to SP (2) nor the basal level of m.c. activity (7) are influenced by pretreatment with atropine.

The result of the present investigation indicates that bradykinin, possibly released during inflammatory conditions in the nose (8), influences m.c. activity via the excitation of SP neurons, mainly unmyelinated C-fibres of trigeminal origin. The final response is probably a result of a combination of release of acetylcholine from parasympathetic nerve fibres and release of SP from sensory fibres. This mechanism could represent a protective reflex in the airway epithelium, activated by irritants (30) or by mediators generated in inflammatory conditions.

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CAPSAICIN STIMULATES MUCOCILIARY ACTIVITY BY RELEASING SUBSTANCE P AND ACETYLCHOLINE

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IV

Abstract

Intraarterial injection of the neuropeptide substance P (SP) accelerates the mucociliary (m.c.) activity. The physiological significance of this finding was investigated using capsaicin, which is known to release SP from unmyelinated C-fibres in sensory nerves.

Capsaicin (3.0-150.0 µg/kg) stimulated m.c. activity in the rabbit maxillary sinus in vivo recorded with a photoelectric technique. This effect was resistant to pretreatment with the adrenergic blocker guanethidine, and to a combination of propranolol and phentolamine. Pretreatment with an SP antagonist ($D\text{-}Pro^{2}$, $D\text{-}Trp^{7,9}$)SP totally inhibited the effect of capsaicin.

The muscarinic antagonist atropine suppressed the initial phase of the response to capsaicin, whereas the response was uniformly suppressed by the ganglionic blocker hexamethonium. These results suggest that capsaicin activates a reflex, via afferent C-fibres containing SP and efferent parasympathetic cholinergic neurons. The final response of the m.c. system reflects the local release of both SP and acetylcholine. The activation of this reflex may represent an important regulation of m.c. activity.

Introduction

The transportation and removal of inhaled particles, bacteria, mucus plugs and cellular debris by mucociliary (m.c.) activity represents a major defence mechanism of the airways. From a recent report it is suggested that inflammatory mediators stimulate m.c. activity. Wanner et al (1), investigated the effect of various inflammatory mediators on ciliary beat frequency (CBF), using sheep tracheal explants. They noticed that CBF was increased by prostaglandins E_1 and E_2, leukotriene-C_4 and by histamine. The mechanisms by which these inflammatory mediators stimulate m.c. activity are not known.

Another agent involved in inflammatory reactions is the neuropeptide substance P (SP). Investigations regarding the neurogenic pathway of inflammation evoked by various irritant chemicals (2), strongly suggest that SP is the mediator of antidromic vasodilation and plasma extravasation which are important components of the inflammatory response (3). Nerve endings with SP-like immunoreactivity are found in the upper airways in the nasal mucosa, sometimes even penetrating the epithelium, in the ganglion cells of the trigeminal ganglion, and in nerve endings in the sphenopalatine ganglion (4, 5, 6). SP-like immunoreactivity is also present in the maxillary sinus and the maxillary nerve in rabbits (7). In the lower airways SP is abundant in the trachea and main bronchi (8, 9).

Intraarterial SP stimulates m.c. activity in the rabbit maxillary sinus, the effect being resistant to atropine (10). The problem of whether the effect of exogenous SP reflects physiological involvement of endogenous neuronal SP in the regulation of m.c. activity can be investigated by the use of SP-releasing drugs such as capsaicin.

Capsaicin induces an acute release of SP-like immunoreactivity from central and peripheral sensory nerve endings (11, 12). However, a recent report suggests that challenge with capsaicin releases not only SP but a related peptide as well, substance K, from lung tissue (13).

It has also been reported that capsaicin induces hypertension after local application in the nose, probably through sympathetic activation (14). Moreover, capsaicin initiates tracheal secretion in dogs through a reflex involving cholinergic effector neurons (15). Since both adrenergic and cholinergic compounds influence m.c. activity in the present experiment model (16, 17), any effect of capsaicin on m.c. activity should be examined with respect to interaction with adrenergic and cholinergic pathways.

A non-cholinergic, non-adrenergic effect of capsaicin on m.c. activity could be explained by release of SP.

The SP antagonist (D-Pro^2, D-Trp^{7,9})SP inhibits the effect of exogenous SP on m.c. activity (18). Thus, if capsaicin influences m.c. activity through release of SP, (D-Pro^2, D-Trp^{7,9})SP could be expected to inhibit the response. On the other hand, if the effect of capsaicin is sensitive to atropine this suggests reflex stimulation of the parasympathetic sphenopalatine ganglion. The ganglionic blocker hexamethonium would be expected to inhibit such a reflex.

Therefore, the aims of the present investigation were to study:

1. The acute effect of moderate doses of capsaicin on m.c. activity.

2. The influence on this effect of the following pharmacological blockades:

   a. Adrenergic blockade by guanethidine, or a combination of phentolamine (α-receptor antagonist) and propranolol (β-receptor antagonist).

   b. Cholinergic blockade of muscarinic receptors by atropine.

   c. Ganglionic blockade with hexamethonium.

   d. Blockade of SP receptors by the SP antagonist (D-Pro^2, D-Trp^{7,9})SP.

Materials and Methods

The experiments were performed on 53 rabbits of both sexes weighing 2.0-3.6 kg. Details of anesthetic and surgical techniques have been published previously (19). The animals were anesthetized with urethane 2 g/kg i.m. as an initial dose, and an extra dose of 0.5 g/kg was given i.v. during the operation. The arterial inlet for drugs was a retrograde cannula in the facial or lingual artery, continuously perfused with saline 2-5 ml/h. The mucosa in the maxillary sinus was exposed through a trepanation hole of about 2x8 mm, which was immediately covered with an antimist window, and sealed with bone wax (Ethicon).

The m.c. activity was studied with the aid of a binocular microscope and the visible transportation of small particles like air bubbles and shed cells was taken as the criterion for a working preparation. One of the eyepieces was then switched to a phototransducer and the m.c. activity recorded by a photoelectric technique (20). The m.c. wave pattern was monitored continuously on an oscilloscope and recorded on an ink writer. The recordings were analysed by a computerized frequency calculator, the m.c. wave frequency being expressed in waves/min. The induced frequency changes were expressed as percentages of the basal m.c. wave frequency (frequency zero level) immediately preceding the administration of the test substances. The m.c. wave frequency was calculated every 10 sec during the experiments, and at intervals of 1-5 min otherwise. ECG and rectal temperature were monitored, and body temperature maintained to 37.0-38.5°C by a heating pad.

The following drugs were tested: Capsaicin, hexamethonium (Sigma, USA), guanethidine and phentolamine (CIBA, Switzerland), propranolol (ICI, UK), atropine (ACO, Sweden) and (D-Pro^2, D-Trp^{7,9})SP (Ferring, FRG, and Ferring, Sweden).

Capsaicin was initially dissolved in saline containing 10% Tween 80 and 10% ethanol, further dilutions were made in saline. The other drugs were dissolved in saline, except (D-Pro^2, D-Trp^{7,9})SP which was dissolved in saline containing 0.5% albumin (Kabi, Sweden) in order to prevent adsorption to glass and plastic surfaces.

Experimental procedure: The m.c. activity was recorded once a minute until the frequency had stabilized for a period of approximately 30 min. The mean value of the frequency variations during the last 5 min preceding an injection was calculated (baseline level) and compared with the frequency zero level, and with the final level 10 min after the

administration. Agreement of these levels indicated that the preparation had remained stable between the different challenges. The test doses were delivered as bolus injections of 0.2 ml during 3 sec in retrograde direction through the arterial cannula, except as described below. Saline with and without 10% Tween 80 + 10% ethanol, served as controls and to reveal any unspecific influence on the preparation, such as drugs remaining in the arterial inlet where they could influence the ensuing experiments.

1. The effect of increasing doses of capsaicin was investigated, and a log dose-response curve was plotted in the interval 0.03-150.0 µg/kg. The time lapse between doses was 10-30 min.

2. Guanethidine 2 mg/kg was given as a bolus injection of 0.5 ml during 10 sec i.a. The effect of guanethidine on m.c. activity was recorded for 15 min following the injection. Guanethidine was given approximately 4 hours (range 189-292 min) before the experiments with capsaicin.

3. Animals were pretreated with propranolol 1 mg/kg i.v. (1 mg/ml during 1 min in an ear vein) followed 10-30 min later by phentolamine 0.2 mg/kg i.a., which was given 1 min before the capsaicin injection.

4. Atropine 0.2 mg/kg was given 2 min before injecting capsaicin or SP. The results were compared with the effect of capsaicin or SP given to the same animal without pretreatment with atropine. These control experiments were generally performed before the experiments with atropine.

5. Hexamethonium 2 mg/kg was given 1 min before injecting capsaicin or SP. The results were compared with the effect of these drugs given to the same animals prior to the experiments with hexamethonium.

6. The SP antagonist (D-Pro^{2}, D-Trp^{7,9})SP 1 mg/kg was given as an intraarterial infusion (1 mg/ml) during 4 min. Capsaicin was given 20 min later. The controls with capsaicin alone were run in the same animals at least 2 hours later.

The doses of the antagonists and the time intervals between the antagonists and the subsequent injections of capsaicin were chosen according to results of previous investigations in the same experiment model (16, 17, 18, 21).

The results were expressed as means $\pm$ standard error of the mean (SEM), if not otherwise stated. The statistical treatment was analysis of variance (one-way, or two-way if appropriate). P-values <0.05 were considered significant.

Results

Capsaicin accelerated the m.c. wave frequency in the dose range 3.0-150.0 µg/kg (corresponding to 10.0 nanomol-0.5 µmol/kg), the maximum response being 40.3 ± 2.8% for a dose of 150.0 µg/kg. The effect was significant compared to saline ($p<0.01$, two-way analysis of variance). The log dose-response curve is shown in Fig. 1 ($p<0.001$ for the whole curve, one-way analysis of variance).

The time-course for the effect of increasing doses of capsaicin on m.c. activity in one rabbit is shown in Fig. 2. An increased m.c. activity appeared very soon after the capsaicin injection, the latency of the response to 30.0 µg/kg capsaicin being 6.3 ± 0.9 sec (n=8). The latency was defined as the time interval between the end of the challenges and a 10% increase in frequency, calculated from the ink writer recordings. Maximal frequency increases occurred within 1 min, and m.c. activity returned to the basal level within a few minutes. The larger the dose, the longer was the duration. If a second dose of capsaicin was given, the effect was virtually identical. For example, when two doses of 30.0 µg/kg were given without any pretreatment to 5 rabbits, the first response was 29.2 ± 3.3%, and the second 27.4 ± 7.1%, $p>0.50$.

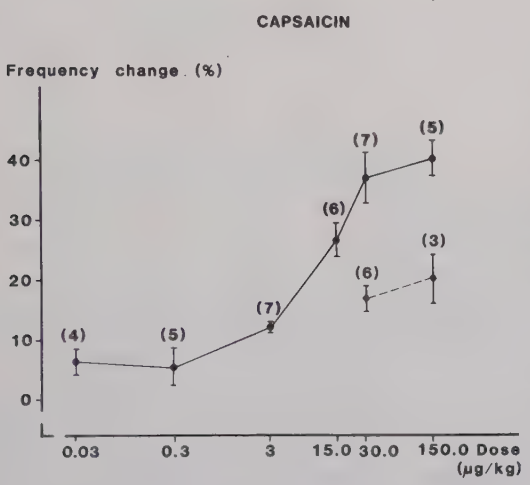

Fig. 1. Effect of various doses of capsaicin on m.c. wave frequency before (●——●) and after atropine 0.2 mg/kg i.a. (◆– –◆). Figures in brackets refer to the number of experiments performed. The results are expressed as means and SEM (standard error of mean). The correlation coefficient for raw data: r=0.79 (n=12). Frequency zero level was 1248 ± 173 waves/min (mean ± SD) before and 1281 ± 133 waves/min after atropine. Note that the entire range of test doses was not given to all animals.

In the dose range studied, neither propranolol, phentolamine nor atropine had any effect on m.c. activity whereas guanethidine suppressed it by -13.0 ± 4.9% 14 min after injection ($p<0.05$, n=6).

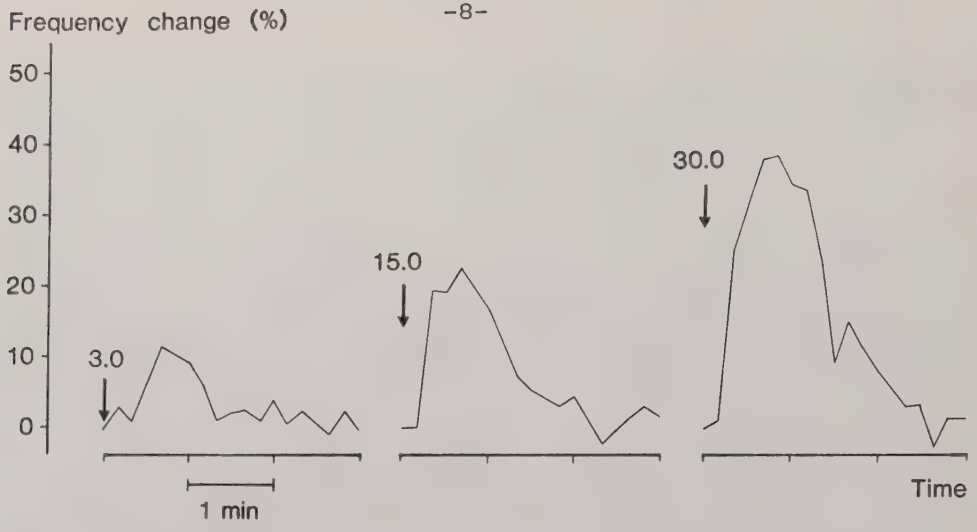

Fig. 2. The effect of 3.0, 15.0 and 30.0 µg/kg capsaicin in one rabbit. The respective frequency zero levels were 1411, 1414 and 1471 waves/min.

Table 1. *The effect of pretreatment with various antagonists on the increase of m.c. activity produced by 30.0 µg/kg capsaicin.*

a.

	n	frequency change	p
Capsaicin (controls)	7	36.8 ± 4.2%	
Guanethidine + capsaicin	6	28.9 ± 2.7%	NS (p=0.14)
Propranolol-phentolamine + capsaicin	6	34.3 ± 2.9%	NS (p=0.66)

The responses are expressed as percentage increase of m.c. wave frequency. Values are means and SEM. Statistical evaluations by the one-way analysis of variance.

b.

	n	frequency change	control experiments	p
Atropine + capsaicin	6	17.0 ± 1.9%	38.0 ± 2.3%	<0.01
Hexamethonium + capsaicin	6	14.8 ± 2.5%	26.1 ± 2.2%	<0.001
($D\text{-}Pro^2$, $D\text{-}Trp^{7,9}$)SP + capsaicin	6	8.4 ± 2.2%	28.2 ± 3.3%	<0.01

The responses are expressed as percentage increase of m.c. wave frequency. Values are means and SEM. Statistical evaluations by the two-way analysis of variance.

Pilo-erection was generally seen in all animals receiving 15.0-150.0 µg/kg capsaicin, indicating a sympathetic activation. However, the effect of capsaicin was not affected by blockade of adrenergic nerves or of adrenoceptors. Neither guanethidine nor the combined pretreatment with propranolol and phentolamine influenced the increase of m.c. activity (see Table 1a).

It was found that atropine weakened the response to capsaicin (see Fig. 1 and Table 1b), the peak responses being halved. Analysis of the time-course for the effect of atropine showed that it inhibited the response to capsaicin during the first minute after injection (see Fig. 3 left). The inhibiting effect of atropine was reproducible and declined as a function of time, as shown in Fig. 4, illustrating the results of repeated experiments in one rabbit.

In contrast, the effect of SP on m.c. activity was not inhibited by atropine, even with respect to the time-course (Fig. 3 right).

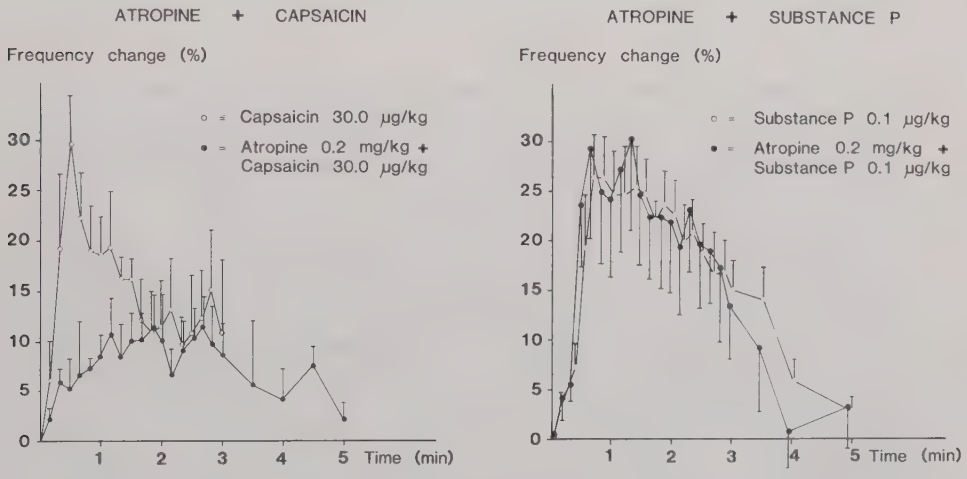

Fig. 3. The time-courses of the effect of pretreatment with 0.2 mg/kg atropine (●——●) on the m.c. response (○——○) to capsaicin 30.0 µg/kg left (n=6), and to 0.1 µg/kg SP right (n=7). The results are expressed as means and SEM. Frequency zero levels were 1309 ± 97 waves/min (capsaicin) and 1202 ± 306 waves/min (SP) (mean ± SD).

The response to capsaicin was also weakened by the ganglionic blocker hexamethonium (see Table 1b). Like atropine, it reduced the peak levels of the increase by about half, but apart from the inhibitory effect of atropine, the response was not delayed. In contrast, hexamethonium pretreatment did not influence the response to SP, the increase of m.c. activity induced by 0.1 µg/kg SP being 36.5 ± 2.4% in hexamethonium pretreated animals, compared to 36.4 ± 4.0% for the control experiments (n=4).

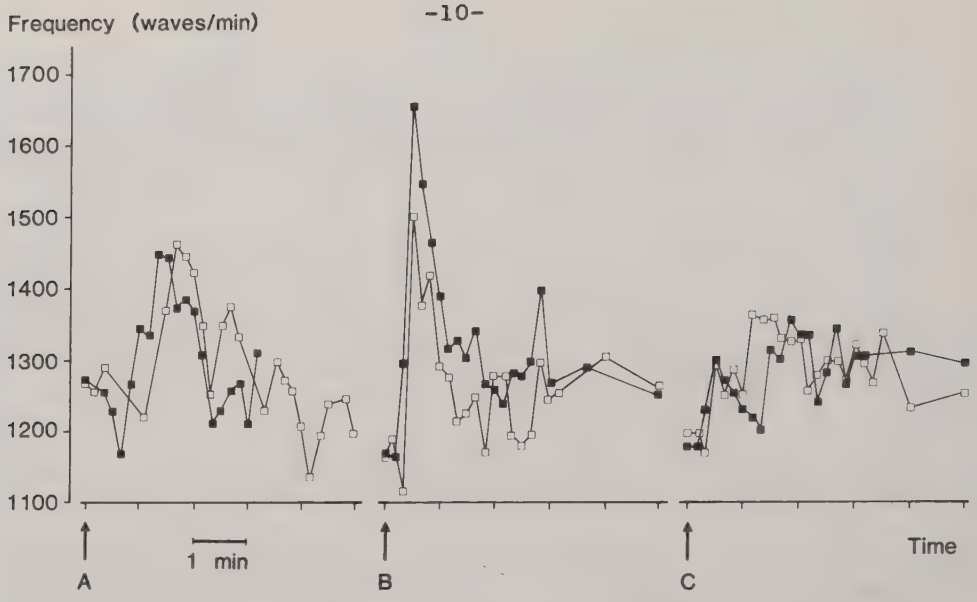

Fig. 4. Three experimental series consisting of two injections of 30.0 µg/kg capsaicin at an interval of 10 min in one rabbit. Pretreatment with 0.2 mg/kg atropine was given 2 min before the series A and C. (□—□) first injection, (■—■) second injection. The time lapse between A and B was three hours, between B and C 30 min.

The SP antagonist (D-Pro^2, D-Trp^{7,9})SP almost totally inhibited the capsaicin-induced stimulation of m.c. activity (Table 1b). In fact, the effect of 30.0 µg/kg capsaicin after SP-blockade (8.4 ± 2.2%) did not differ from the saline controls (4.7 ± 1.5%) in the same animals. The time course of the response to capsaicin with and without pretreatment with the SP antagonist is illustrated in Fig. 5.

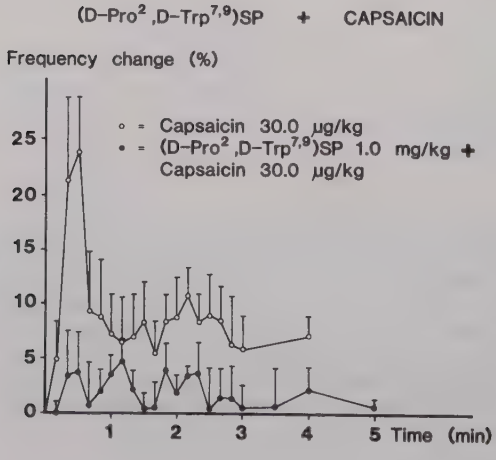

Fig. 5. The time-course of the effect on m.c. activity of 30.0 µg/kg capsaicin in animals pretreated with 1 mg/kg (D-Pro^2, D-Trp^{7,9})SP (●—●) (n=4). Control experiments (○—○) in the same animals more than 2 hours later. Frequency zero level was 1310 ± 267 waves/min (mean ± SD).

Discussion

Capsaicin 3.0-150.0 µg/kg (corresponding to 10 nanomol-0.5 µmol/kg) caused a dose-dependent increase of m.c. wave frequency in the rabbit maxillary sinus (Figs. 1 and 2). The stimulation of m.c. activity had a short latency, and no overt tachyphylaxis was seen.

The maximum response recorded, an increase of about 40%, is comparable to the effects of SP and methacholine on the same preparation (an increase of about 50% (10, 17)) and stronger than the maximum response to the β_2-agonist salbutamol, which increases m.c. activity by about 25% in the same experiment model (16).

Previous investigations have shown that the m.c. activity in the rabbit maxillary sinus is influenced by drugs acting on parasympathetic as well as sympathetic receptors (16, 17). In the present study, pilo-erection indicating sympathetic activation was noted after challenge with capsaicin. The effect of sympathetic stimulation on m.c. activity is complex, α-agonists causing deceleration and β_2-agonists causing acceleration (16). However, pretreatment with sympathetic blockers like guanethidine, or a combination of phentolamine and propranolol, did not influence the m.c. response to capsaicin. Thus, the sympathetic activation induced by capsaicin does not seem to be important for the m.c. activity.

The effect of capsaicin apparently involves cholinergic pathways (see Figs. 3 and 4). An explanation to this interaction was suggested by Davis et al (15), who studied the in vivo effect of intraarterial capsaicin on canine tracheal gland secretion. It was found that 3 µg capsaicin increased the activity in these glands. However, the response was totally inhibited by atropine. Since capsaicin stimulates afferent vagal C-fibres (22), it was concluded that they constituted the afferent pathway of a secretory reflex mediated by cholinergic effector neurons. The present findings support this concept. The afferent pathway of the reflex arc consists of unmyelinated C-fibres that probably release SP, or an SP-like peptide after challenge with capsaicin both at the peripheral (12) and central nerve endings (11). A release of SP is supported by the effect of the SP antagonist (D-Pro^2, D-Trp^{7,9})SP on the response to capsaicin (see Fig. 5).

The efferent pathway of the reflex arc is important for the m.c. response to capsaicin as well, since the effect of capsaicin was partly inhibited by atropine. In particular, the rapid initial response disappeared completely after atropine pretreatment (see Figs. 3 and 4), and was therefore in all probability mediated by

release of acetylcholine. The finding that the ganglionic blocker hexamethonium did not inhibit the initial response to capsaicin suggests that SP released from stimulated C-fibres, besides the more likely reflex activation, could stimulate cholinergic neurons directly, as seen for SP in parotid gland tissue (23). The best way to evaluate the cholinergic response to capsaicin requires severing the preganglionic parasympathetic nerve (the Vidian nerve) or removing the sphenopalatine ganglion. Unfortunately, these procedures have so far been technically impossible to accomplish in the rabbit without interfering with the maxillary nerve, which carries sensory fibres from the sinus. Interestingly, the effect of exogenous SP was not influenced by either atropine or hexamethonium, suggesting any effect of capsaicin on m.c. activity that is resistant to pretreatment with these drugs is mediated by release of SP (or a related peptide). The putative mechanisms regulating m.c. activity discussed in the present investigation are summarized in Fig. 6.

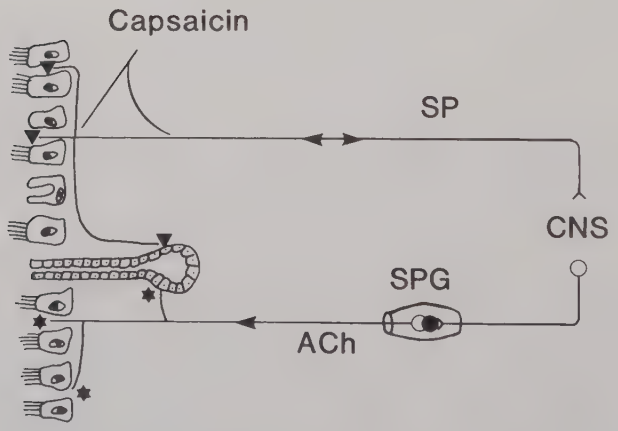

Fig. 6. Model for a protective reflex regulating m.c. activity in rabbit maxillary sinus. Capsaicin stimulates SP-containing C-fibres in the epithelial and subepithelial layer. The m.c. activity is increased by release of SP from the peripheral endings, and by release of acetylcholine (ACh) from cholinergic (parasympathetic) effector neurons. Arrows ⟶ indicate the direction of impulse propagation. ⟷ symbolizes the dual sensory-efferent function of SP-containing neurons. Possible points of action for the antagonists are indicated. ▼ = (D-Pro^2, D-Trp^{7,9})SP, ✸ = atropine, and ● = hexamethonium. SPG = sphenopalatine ganglion.

The SP-releasing property of capsaicin has been used for pharmacological studies of SP-mediated effects in the airways. Intraarterial infusion of capsaicin to cats induced a vasodilatory response in the nose (24), while topical application of capsaicin or SP to the nasal mucosa in rats caused a significant increase in Evans blue extravasation which is considered to represent increased vascular permeability (25). The results of these studies indicate that SP neurons mediate other protective mechanisms in the upper airways than stimulation of m.c. activity.

When human subjects inhaled moderate doses of capsaicin coughing was the main response (26). There was no influence on measurements of FEV afterwards. In contrast to this, capsaicin and SP caused contraction of human bronchi in vitro, which was resistant to atropine and the H_1-receptor antagonist mepyramine (27). However, the maximum response to SP was comparatively weaker than that of acetylcholine or histamine in the same preparations. These results differed from experiments on guinea-pig trachea, where SP and capsaicin were much more potent. This indicates either a species difference, or reflects the fact that the human bronchi came from elderly patients with a history of smoking and bronchitis, who underwent surgery for pulmonary tumors. Whether such a difference influences the conclusions that can be drawn from the present investigation regarding the regulation of m.c. activity in homo, remains an open question. Also, as our maxillary sinus model has no counter-part in the lower respiratory tract at present, conclusions regarding the effect of capsaicin on m.c. activity in the lower airways will have to await further confirmation.

At the moment the hypothesis that the capsaicin-induced acceleration of m.c. activity is due to release of SP, or an SP-like mediator from sensory fibres, and release of acetylcholine from parasympathetic neurons seems the most probable. However, it must be remembered that capsaicin also releases other neuropeptides like vasoactive intestinal polypeptide (VIP), cholecystokinin-octapeptide (CCK8) and somatostatin (28, 29). VIP does not influence m.c. activity (10), while the effect of CCK8 and somatostatin on m.c. activity is unknown. On the other hand, the latter peptides have not been observed in the upper airways. As the conclusions in the present investigation are supported by the inhibitory effect of (D-Pro^2, D-Trp^{7,9})SP on the m.c. response to capsaicin, it must be emphasized that this antagonist also inhibits the stimulating effect on m.c. activity of the related peptide substance K (Lindberg & Mercke, unpublished results). Release of substance K after injection of capsaicin has been shown in guinea-pig lung tissue (13).

The present finding that capsaicin stimulates m.c. activity indicates that endogenously released SP (or an SP-like mediator) may influence m.c. activity in certain conditions. For example, C-fibre receptors in the lower airways are stimulated not only by capsaicin but also by inhaled irritants such as cigarette smoke and sulphur dioxide, and by endogenous mediators like

histamine, bradykinin and prostaglandins (30). Thus, the acceleration of m.c. activity by the release of SP from sensory nerve endings, may be part of a protective reflex mechanism in the airways, activated under many different conditions. The reflex arc includes the release from parasympathetic neurons of acetylcholine, also known to accelerate m.c. activity. This protective reflex may be of major importance in the regulation of m.c. activity. Interesting support for this aspect is provided by Gamse et coworkers (28) who reported that most of the rats that died after neonatal capsaicin treatment given to deplete SP (the mortality was about 50% within 3-5 weeks), showed extensive atelectatic areas in the lungs, a condition which is associated with impaired m.c. transportation and accompanying stagnation of mucus plugs.

Acknowledgements

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ANTIDROMIC NERVE STIMULATION ACCELERATES MUCOCILIARY ACTIVITY IN RABBIT MAXILLARY SINUS

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Abstract

The neuropeptide substance P (SP) is released from unmyelinated C-fibres in sensory nerves by antidromic nerve stimulation. In order to study the effect on mucociliary (m.c.) activity, the isolated distal end of the maxillary nerve was stimulated electrically (8 V, pulse rate 10 Hz, 5 ms pulses) for 2-4 min, while the m.c. wave frequency in the maxillary sinus was recorded by a photoelectric technique.

The m.c. wave frequency increased during stimulation by 35.4 $\pm$ 4.9% (range 22.0-62.2%). Following pretreatment with atropine the m.c. activity increased by only 20.0 $\pm$ 1.3% (range 14.9-21.9%) indicating that part of the response was mediated via stimulation of muscarinic receptors. The cholinergic part of the response is presumably secondary to release of SP since the SP antagonist (D-Pro^{2}, D-Trp^{7,9})SP abolished the effect of nerve stimulation. Sympathetic ganglionectomy did not influence the response to antidromic nerve stimulation. It is concluded that antidromic stimulation of the maxillary nerve accelerates m.c. activity through a dual mechanism, involving release of SP, or SP-like peptides, from sensory C-fibre nerve endings as well as release of acetylcholine.

Introduction

Mucociliary (m.c.) activity is accelerated in the rabbit maxillary sinus following i.a. injection of the neuropeptide substance P (SP) (1). The effect of SP is resistant to the muscarinic antagonist atropine and the ganglionic blocker hexamethonium (1, 2), suggesting that the effect of SP is direct. SP-like immunoreactivity is found in nerve fibres of trigeminal origin in the subepithelial layer of the nasal mucosa, sometimes even penetrating into the epithelium, and in nerve endings in the sphenopalatine ganglion (3, 4, 5), and also in nerve fibres in the mucosa of the maxillary sinus and in the maxillary nerve of rabbits (Lindberg et al, unpublished observations). In the lower airways, SP is abundant in the trachea and main bronchi (6, 7).

The SP-containing nerve fibres are probably sensory and of unmyelinated C-fibre type (8), a view supported by the finding that the number of fibres containing SP in the nasal mucosa is markedly reduced by treatment with large doses of capsaicin (5). Capsaicin treatment is known to deplete SP from peripheral sensory nerves (9), and to cause degeneration of chemosensitive sensory neurons (10). However, it is quite possible that C-fibres may contain other SP-related peptides, since capsaicin also releases substance K (SK) from guinea pig lung tissue (11). Also, the coexistence of SP and a third peptide, calcitonin gene-related peptide (CGRP) has been demonstrated in capsaicin--sensitive sensory neurons (12).

C-fibre endings in the lower airways are stimulated by bradykinin (13), a tentative inflammatory mediator, and by small doses of capsaicin (14). The effects of SP on m.c. activity in the upper airways is mimicked by i.a. injection of these drugs (2, 15). However, the rapid initial effects of bradykinin and capsaicin on m.c. activity are suppressed by atropine, suggesting that C-fibre activation either stimulates a reflex arc involving cholinergic effector neurons (16) or has a direct effect on cholinergic neurons. The cholinergic acceleration is probably secondary to release of SP, since the effects of both bradykinin and capsaicin on m.c. activity are blocked by the SP antagonist (D-Pro^{2}, D-Trp^{7,9})SP, which inhibits the effect of exogenous SP on m.c. activity (17).

An alternative to the pharmacological activation of C-fibres is antidromic nerve stimulation of peripheral sensory neurons which was originally performed on spinal dorsal roots (18). Thus, release of SP-like immunoreactivity from the dental pulp has been demonstrated after stimulation of the isolated inferior alveolar nerve (19). Furthermore, the vasodilation in the dental

pulp caused by antidromic trigeminal nerve stimulation is inhibited by an SP antagonist (20). In the upper airways, antidromic nerve stimulation causes vasodilation (21) and plasma-extravasation (22) probably a consequence of the release of SP.

Electrical stimulation of the isolated maxillary nerve, which carries sensory nerve fibres to the maxillary sinus, would be expected to increase m.c. activity by release of SP from unmyelinated C-fibres. However, previous results of pharmacological C-fibre stimulation show that part of the response is mediated by an atropine-sensitive (cholinergic) effect (2, 15). It has also been suggested (23) that adrenergic responses may dominate in the nasal mucosa after maxillary nerve stimulation. Since both cholinergic and adrenergic compounds influence m.c. activity in the present experiment model (24, 25), possible cholinergic and adrenergic involvement in the antidromic nerve stimulation must be evaluated.

Therefore, the aims of the present investigation were to study:

1. The effect on m.c. activity by antidromic stimulation of the maxillary nerve.

2. The influence of the following pretreatments on electrically stimulated m.c. activity:

   a. Blockade of muscarinic receptors by atropine.

   b. Blockade of SP receptors by the SP antagonist ($D\text{-}Pro^{2}, D\text{-}Trp^{7,9}$)SP.

   c. Cervical sympathectomy.

Materials and Methods

The experiments were performed on 22 rabbits of a white strain of both sexes weighing 1.8-3.2 kg. Details of anesthetic and surgical techniques have been published previously (26). The animals were anesthetized with urethane 2 g/kg i.m. as an initial dose, and an extra dose of 0.5 g/kg was given i.v. during the operation. Since SP neurons probably carry nociceptive impulses (27), and since release of SP by any painful stimulus during preparation could be expected to influence the results of the ensuing nerve stimulation, the urethane was supplemented by local anesthesia given as 1-2 drops of oxibuprocaine 0.4% on the cornea, and 1 ml of lidocaine 1.0% (Xylocain, Astra, Sweden) which was infiltrated around the skin incision below the lower eyelid.

The mucosa in the fronto-superior part of the maxillary sinus was exposed through a trepanation hole of about 2x8 mm which was immediately sealed with an antimist window and bone wax (Ethicon). In five animals the facial artery or lingual artery was cannulated for injections of drugs, and the cannula continuously perfused with physiological saline 2-5 ml/h in order to keep the inlet open.

The maxillary nerve was exposed by a modified version of the technique described by Änggård (28). The eyeball was opened, emptied, retracted upwards and stay-sutured. The bottom of the orbit was then carefully dissected through an incision in the lower eyelid. The maxillary nerve courses medially to the alveolar tube and the sphenopalatine ganglion in rabbit occurs as a thin leaf-like structure medially to the nerve (29). The nerve was isolated and cut high in the orbit, and the distal end was placed on a bipolar platinum electrode. Possible connections between the ganglion and the maxillary nerve (29) might be damaged during this manoeuvre. The cut nerve was covered with Plastibase (Squibb) or cotton soaked with saline and stimulated with monophasic, rectangular wave pulses of different voltage, rate and duration from a Grass S48 stimulator. An isolation unit SIU5 was connected between the stimulator and the rabbit. In preparatory experiments the voltage and current actually delivered to the nerve were determined with the aid of a resistance-bridge, so that any losses (especially in the SIU5) could be compensated for.

The m.c. activity was observed through a binocular microscope, and the transportation of small particles like mucus clumps and shed cells was the criterion for a working preparation. One eyepiece was then switched to a phototransducer and the m.c. activity recorded by a photoelectric technique (30). The

recordings were analysed by a computerized frequency calculator which calculated the m.c. wave frequency in waves/min every 10 sec during the experiments. The induced frequency changes were expressed as percentages of m.c. wave frequency (frequency zero level) immediately preceding nerve stimulation. The mean value of the frequency variations during the 5 min preceding a nerve stimulation was calculated (baseline level) and compared with the frequency zero level, and with the final level 10 min after the nerve stimulation. ECG and rectal temperature were monitored, and body temperature adjusted to 37.0-38.5 °C with the aid of a heating pad.

(D-Pro^2, D-Trp^{7,9})SP was dissolved in physiological saline containing 0.5% albumin (Kabi, Sweden) in order to avoid adherence to the walls of glass and plastic cannulas, while methacholine (Sigma, USA) and atropine sulphate (ACO, Sweden) were dissolved in saline.

Experimental procedure. After isolating the maxillary nerve, the maxillary sinus was trepanated and the m.c. activity recorded. Nerve stimulation was not commenced until the frequency had been stable for approximately 30 min.

In preparatory experiments rabbits were stimulated continuously for 2 min with 5 ms pulses at a rate of 10 Hz and a voltage which varied between 4-10 V. A few of these rabbits showed weak responses to amplitudes of 4-6 V, whereas all animals responded to 8 V. This stimulation level was therefore chosen for the subsequent experiments. A further increase to 10 V did not evoke a stronger response.

The following experimental series were then performed:

1. Nine rabbits were stimulated continuously for 2 min without pretreatment of any kind. Impulses were generated with a voltage of 8 V, a pulse rate of 10 Hz and a pulse duration of 5 ms. Stimulations were repeated at 30-60 min intervals. The first 4 rabbits in this series were initially stimulated with a low intensity stimulation (31) (2 V, 10 Hz, 0.2 ms).

2. The effect of pretreatment with 0.2 mg/kg atropine (volume 0.2 ml) injected 5 min before nerve stimulation, and pretreatment with a 4 min 1.0 mg/kg infusion of the SP antagonist (D-Pro^2, D-Trp^{7,9})SP (1.0 mg/ml) 10 min prior to nerve stimulation was investigated in 5 rabbits, using the maxillary sinuses and nerves on both sides. An interval of 3-5 hours elapsed before the experiment was started on the other side. The rabbits were stimulated with impulses of 8 V, 10 Hz, 5 ms for 4 min. The effect of these two antagonists could be compared on the same animal, since the duration of effect of the SP antagonist has been shown not to exceed 1-2 hours (17). Muscarinic receptors were challenged with 0.5 µg/kg methacholine (volume 0.2 ml) injected 10 min after nerve stimulations.

3. Cervical sympathectomy was performed in 5 rabbits by extirpation of the superior cervical ganglion under mebumal anesthesia (30-40 mg/kg i.v.) two weeks prior to nerve stimulation (8 V, 10 Hz, 5 ms for 4 min).

4. The intact maxillary nerve was stimulated in 3 rabbits with impulses of 8 V, 10 Hz, 5 ms for 4 min.

The results are expressed as means and SEM (standard error of the mean), if not otherwise stated. Since it was not clear that the results had a Gaussian distribution (were normally distributed), the medians and ranges are also reported. Statistical treatment included the non-parametric Mann-Whitney U test (unpaired data) and the randomization test for matched pairs (paired data) (32). P-values <0.05 were considered significant.

Results

Low intensity (2 V, 10 Hz, 0.2 ms) stimulation did not significantly increase m.c. activity (7.3 ± 1.6%, n=4) whereas all animals in the untreated group responded to pulses of 8 V, 10 Hz and 5 ms. The acceleration of m.c. activity in these rabbits was 35.4 ± 4.9% (median 30.0%), see Table I.

Repeated stimulations gave varying responses. In some rabbits results were reproducible, but in the majority (6/9) the second response was lower.

The response occurred immediately upon stimulation in some rabbits (3/9) (Fig. 1a), but usually there was a short latency (median 30 sec, range 10-70 sec, Fig. 1b). The duration of the response varied (Fig. 1a and 1b, Table I), but in most cases (7/9) m.c. wave frequency returned to baseline levels within 10 min.

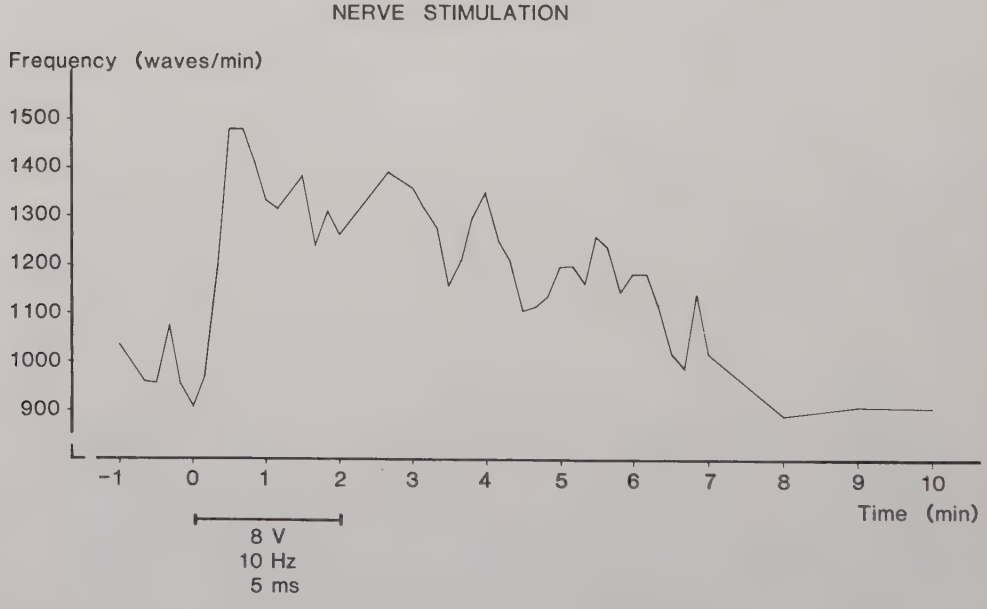

Fig. 1a. An example of the effect of antidromic nerve stimulation in a rabbit that responded immediately with an increase of m.c. wave frequency. Rabbit No 2 in Table I.

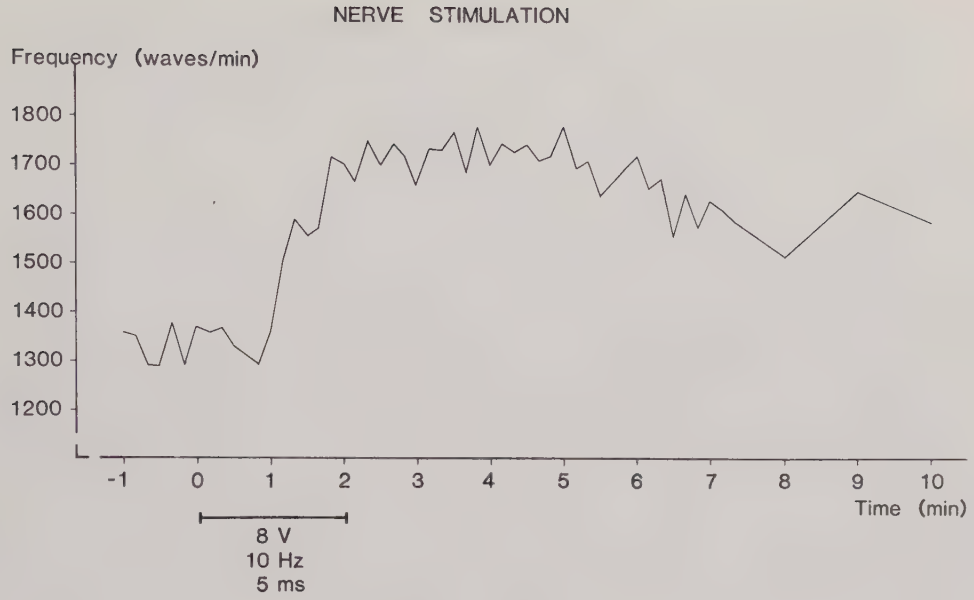

Fig. 1b. An example of the effect of antidromic nerve stimulation in a rabbit that responded after a delay. Rabbit No 9 in Table I (the median rabbit in the untreated group).

Table I. *The effect of antidromic nerve stimulation (8 V, 10 Hz, 5 ms) on m.c. wave frequency*

Rabbit	Baseline level	Zero level	Max response	Final level
1	1134 ± 24	1152	1477 (27.9%)	1078
2	984 ± 60	912	1479 (62.2%)	1011
3	1204 ± 17	1212	1689 (39.4%)	1383
4	1165 ± 95	1146	1785 (55.8%)	984
5	1206 ± 69	1191	1464 (22.9%)	1219
6	1667 ± 39	1662	2061 (24.0%)	1642
7	1213 ± 38	1230	1659 (34.9%)	1258
8	1242 ± 50	1173	1431 (22.0%)	1222
9	1363 ± 10	1368	1779 (30.0%)	1513

Maximum frequency increase: Mean 35.4 ± 4.9%. Median 30.0% (range 22.0-62.2%).

Baseline level refers to the mucociliary wave frequency in waves/min (means ± SD) during 5 min immediately preceding the nerve stimulation, zero level is the frequency at the time of stimulation and final level is the frequency 10 min after the end of the nerve stimulation. Max response figures in brackets are the percentage increase induced by nerve stimulation, and used for the statistical calculations.

Pretreatment with 0.2 mg/kg atropine reduced the response to antidromic nerve stimulation in particular during the first 2 min (Fig. 2 and Table IIa), $p<0.01$. The peak increase of m.c. activity was reduced to 20.0 ± 1.3% (median 21.3%), while the latency was not significantly prolonged, median 40 sec (range 20-100 sec). Methacholine injected after these nerve stimulations had no effect on m.c. activity.

The SP antagonist (D-Pro^2, D-Trp^{7,9})SP abolished the response to antidromic nerve stimulation (Fig. 2 and Table IIb), $p<0.01$. The inhibition was also significant compared with atropine pretreatment, $p<0.05$. Repeated stimulations (4 min each) gave non-significant responses (in one of the rabbits - No 11 - a small increase was seen during one min). In cases where atropine had been administered to the first investigated side, injections with 0.5 μg/kg methacholine 3-5 hours later produced the expected acceleration of m.c. activity on the contralateral side, indicating that muscarinic receptors on this side were not influenced by the atropine.

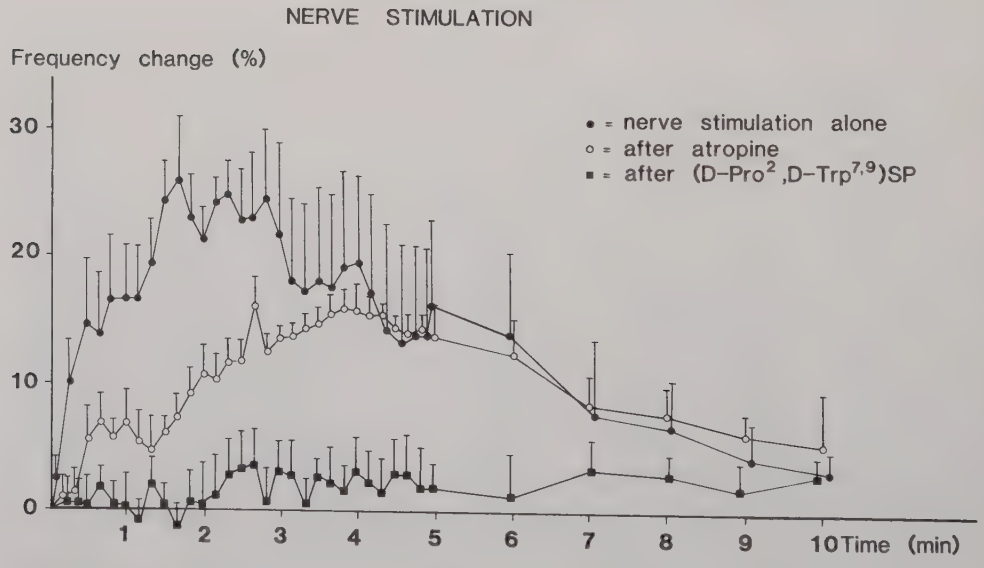

Fig. 2. The effect of pretreatment with 0.2 mg/kg atropine (○——○, n=5) and pretreatment with 1.0 mg/kg (D-Pro^2, D-Trp^{7,9})SP (■——■, n=5) on the m.c. response to antidromic nerve stimulation. Note that nerve stimulation alone (●——●, n=5) was given for 2 min, while pretreated animals were stimulated for 4 min. Frequency zero level was 1272 ± 129 waves/min (mean ± SD).

Table II.

a. The effect of pretreatment with 0.2 mg/kg atropine given 5 min prior to nerve stimulation (8 V, 10 Hz, 5 ms)

Rabbit	Baseline level	Zero level	Max response	Final level
10	1258 ± 52	1206	1470 (21.9%)	1249
11	1329 ± 72	1449	1758 (21.3%)	1677
12	1269 ± 45	1275	1551 (21.7%)	1548
13	1318 ± 14	1326	1524 (14.9%)	1275
14	1124 ± 26	1104	1329 (20.4%)	1263

Maximum frequency increase: Mean 20.0 ± 1.3%. Median 21.3% (range 14.9-21.9%)

b. The effect of pretreatment with 1.0 mg/kg ($D\text{-}Pro^2$, $D\text{-}Trp^{7,9}$)SP given 10 min prior to nerve stimulation (8 V, 10 Hz, 5 ms)

Rabbit	Baseline level	Zero level	Max response	Final level
10	1221 ± 37	1290	1320 (2.3%)	1306
11	1408 ± 33	1458	1608 (10.3%)	1573
12	1399 ± 21	1374	1452 (5.7%)	1440
13	1416 ± 23	1390	1476 (6.2%)	1288
14	1196 ± 33	1227	1278 (4.2%)	1242

Maximum frequency increase: Mean 5.7 ± 1.3%. Median 5.7% (range 2.3-10.3%).

Sympathetic ganglionectomy did not affect the response to antidromic nerve stimulation (Fig. 3 and Table III). The median latency of the response was 40 sec (range 20-120 sec), not different from untreated rabbits.

Three rabbits with an intact nerve were stimulated. One of these rabbits did not respond, while the m.c. wave frequency in the two others was increased by 22.0% and 14.3%, respectively. All three animals reacted with a brief period of apnea immediately upon stimulation, followed by an increased respiratory frequency.

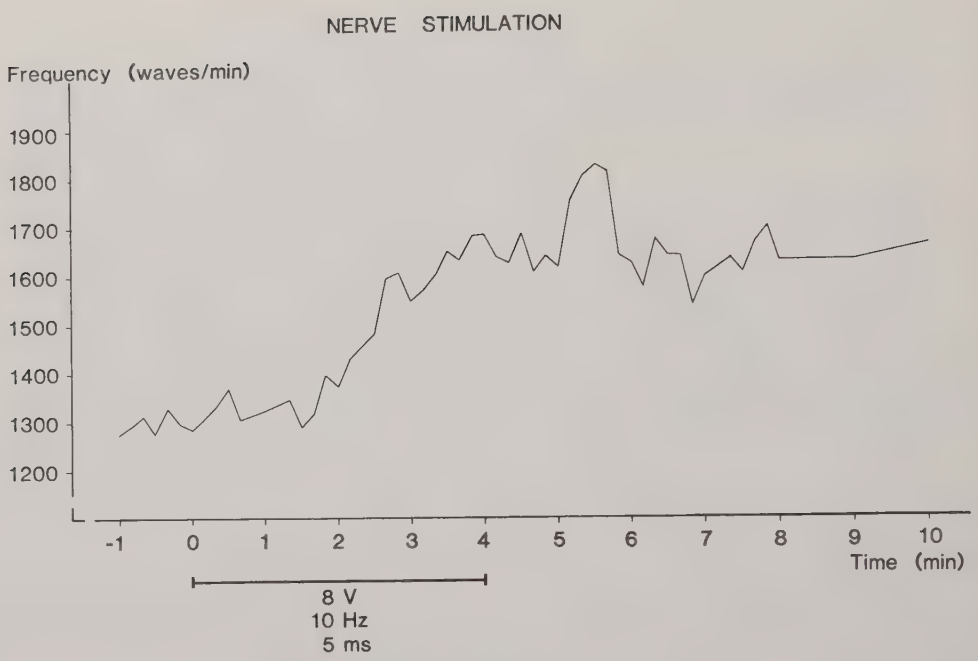

Fig. 3. An example of the effect of antidromic nerve stimulation in a rabbit that had been sympathectomized two weeks prior to the experiment. Rabbit No 16 in Table III (the median rabbit in the sympathectomized group).

Table III. *The effect of sympathectomy two weeks prior to nerve stimulation (8 V, 10 Hz, 5 ms)*

Rabbit	Baseline level	Zero level	Max response	Final level
15	1252 ± 65	1188	1773 (49.2%)	1620
16	1334 ± 59	1284	1698 (32.2%)	1639
17	1537 ± 91	1410	1692 (20.0%)	1384
18	1262 ± 68	1272	1572 (23.6%)	1548
19	1213 ± 55	1191	1857 (55.9%)	1575

Maximum frequency increase: Mean 36.2 ± 7.0%. Median 32.2% (range 20.0-55.9%).

Discussion

Antidromic nerve stimulation of the isolated maxillary nerve accelerated the m.c. activity in the rabbit maxillary sinus by about 30% in untreated and in sympathectomized animals (Tables I and III). Since the maximum frequency increase over prestimulatory activity declined to about 20%, and the initial response in particular was suppressed after pretreatment with atropine (Table IIa and Fig. 2), it was obvious that part of the response was mediated by an effect on muscarinic receptors, probably via release of acetylcholine from as yet unidentified nerve fibres. A similar partly cholinergic response to antidromic nerve stimulation of a branch of the trigeminal nerve (the mental nerve) was reported by Couture et al (33).

However, the cholinergic component of the response was probably indirect. It was not possible to accelerate m.c. activity by low intensity stimulation (31) which is known to stimulate cholinergic (parasympathetic) effector neurons. Such stimulation of the Vidian nerve is known to produce vasodilation in the cat nasal mucosa after sympathetic ganglionectomy (21). Further support for indirect cholinergic involvement in the stimulation of m.c. activity was provided by the effect of the SP antagonist (D-Pro^{2}, D-Trp^{7,9})SP which abolished the response to antidromic nerve stimulation.

The maximum frequency increase over prestimulatory levels was about 20% (range 14.9-21.9%) after treatment with atropine. The small variation of this response is in contrast to the larger variation of the response in the other groups (Tables I, II, III and Fig. 2). Interestingly, an increase of m.c. activity of about 20% is also found after atropine pretreatment of rabbits whose C-fibres instead are activated pharmacologically by capsaicin (2).

Atropine itself does not influence basal m.c. activity (24), but it cannot be ruled out that atropine pretreatment changes the composition of the mucus and its rheological properties. A more viscous secretion might in turn inhibit rapid responses in the m.c. system. Thus, the parasympathomimetic drug pilocarpine increases mucin output from the rabbit nasal mucosa (34), suggesting that cholinergic neurons may participate in the regulation of mucus secretion in the rabbit nose. On the other hand, the effect of SP on m.c. activity is resistant to atropine, including the time-course of the response (1, 2), making this explanation of the effect of atropin pretreatment less likely.

The finding that sympathectomy did not have any major influence on the response to antidromic nerve stimulation is in agreement with the effect of adrenergic blockade in previous experiments with capsaicin (2). In fact, one would expect a stronger response in sympathectomized rabbits, because SP effects and sympathetic activity sometimes counteract each other (35, 36). Possibly, this counteraction is the explanation for the rather weak effect of nerve stimulation in the rabbits with an intact maxillary nerve. However, a more complete evaluation of the interactions between sympathetic activation and SP on m.c. activity needs further experiments, for example stimulation of the superior cervical ganglion.

The possibility that antidromic nerve stimulation also releases SK and CGRP besides SP cannot be ruled out (11, 12). However, preliminary results with SK indicate that effects on m.c. activity are similar to SP, including inhibition of the response by (D-Pro^{2}, D-Trp^{7,9})SP, while CGRP does not influence m.c. activity (Lindberg & Mercke, unpublished results). Thus, release of SK may explain the results of antidromic nerve stimulation, which is the reason why we prefer to describe "SP, or SP-like peptides" as the cause of the increase of m.c. activity after antidromic nerve stimulation.

Previous investigations regarding an accelerating effect of nerve stimulation on m.c. activity or transport are few, presumably due to technical difficulties. For example, a simultaneous muscular contraction in the lower airways during stimulation might impair the recordings. However, Morley & Sanjar (37) reported that m.c. transport in the frog oesophagus was improved after electrical stimulation of the vagus nerve and that the response was inhibited by atropine and hexamethonium. Their stimulation range was a "low intensity stimulation" since increasing the voltage above 1.5 V produced muscular contractions that disturbed the recording procedure. Other results have been obtained using the frog palate as a model. Seo (38) reported that electrical stimulation of the glossopharyngeal nerve stimulated a reflex with increased m.c. transport mediated via efferents in the palatine nerve. This reflex was further investigated by Slaughter & Aiello (39), who found that mucus secretion from goblet cells, m.c. transport and ciliary beating increased after nerve stimulation with impulses of 1.5 V, 10 Hz, 0.8 ms. These effects were markedly reduced by atropine.
A similar reflex could be involved in the regulation of m.c. activity also in the rabbit maxillary sinus (2), but the reflex is probably mediated both via the central nervous system and via local mechanisms. The hypothesis of local regulation of m.c. activity is supported by the present investigation showing that C-fibre activation, probably releasing SP, is primary to cholinergic influences on m.c. activity in sensory denervated mucosa. Also, the effect of nerve stimulation in animals with an intact maxillary nerve was not stronger than in animals with sectioned maxillary nerve. The local reflex mechanism involves release of SP (at C-fibre endings) and acetylcholine, both increasing m.c. activity. The reflex may be mediated via the sphenopalatine ganglion, which could be argued since SP-like

immunoreactivity is present in nerve endings close to the ganglion cells in the sphenopalatine ganglion (5), or via direct stimulation of cholinergic neurons (40, 41). This question cannot be settled at present because it was not possible to separate the connections between the maxillary nerve and the sphenopalatine ganglion with certainty.

The present investigation gives further evidence of a physiological role for endogenous SP, or SP-like peptides, in unmyelinated C-fibres in the regulation of m.c. activity. C-fibres in the lungs are activated by many different chemical agents, for example by inhaled irritants such as ammonia, volatile anesthetics like ether, poison gases and even endogenous mediators like histamine, acetylcholine, serotonin and prostaglandins, see Widdicombe (42). It seems that C-fibre endings are primarily stimulated by tissue damage and by release of mediators. Thus the increase of m.c. activity produced by release of SP from sensory nerve endings and by acetylcholine from cholinergic neurons might constitute an important protective mechanism in the airway epithelium.

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THE MORPHOLOGICAL BASIS FOR THE EFFECT OF SUBSTANCE P ON MUCOCILIARY ACTIVITY IN RABBIT MAXILLARY SINUS

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VI

Abstract

The mucociliary (m.c.) activity in rabbit maxillary sinus is accelerated by the neuropeptide substance P (SP). The morphological basis for this mechanism was investigated by immunocytochemistry using monoclonal antibodies directed against SP.

SP-like immunoreactivity was found in nerve fibres in the subepithelial layer of the sinus mucosa, in the maxillary nerve, and in nerve endings close to nerve cell bodies in the sphenopalatine ganglion. Thus, there is morphological evidence in the rabbit that SP plays a role in the regulation of m.c. activity. The results also support the view that the rabbit maxillary sinus is a suitable experimental model for studying SP effects in the airway mucosa.

Introduction

Immunocytochemical studies have shown that the tachykinin substance P (SP) occurs in nerve fibres in the upper respiratory tract of several species. The fibres are seen around blood vessels, seromucous glands and in the subepithelial layer, sometimes even within the epithelium. SP-immunoreactive nerve fibres and occasional nerve cell bodies are also found in the sphenopalatine ganglion (1). The majority of the fibres originate in the trigeminal ganglion, where SP-positive nerve cell bodies are numerous (1, 2, 3).

The SP-immunoreactive nerve fibres probably constitute sensory endings of unmyelinated C-fibres, since treatment with high doses of capsaicin depletes SP from peripheral sensory nerve endings (4) and causes degeneration of chemosensitive sensory afferents (5), especially unmyelinated C-fibres (6). Recently it was reported that capsaicin also releases another tachykinin, substance K (SK), from guinea pig lung tissue, suggesting that C-fibres may contain other tachykinins as well (7).

Investigations regarding the neurogenic pathway of inflammation produced by antidromic nerve stimulation or evoked by various irritant chemicals (8) suggest that SP mediates this mechanism (9) by release from the peripheral ending. Thus, SP in the upper airways seems to mediate neurogenic protective mechanisms such as vasodilation and plasma extravasation (10, 11). Also, reflexes like sneezing on exposure to irritants are activated by stimulation of capsaicin-sensitive C-fibres (12).

Another protective function in the upper airways mediated by SP neurons is stimulation of the mucociliary (m.c.) activity. In the rabbit maxillary sinus, SP causes a strong increase of m.c. wave frequency (13), well comparable to the effect of the parasympathomimetic drug methacholine (14). This effect of SP is resistant to atropine. The accelerating effect of exogenous SP could be mimicked by low doses of capsaicin or bradykinin (15, 16) which stimulate C-fibres in the airways (17, 18). However, in contrast to the effect of SP, the stimulating effect of these compounds was partly inhibited by atropine. One explanation for the discrepancy between the effect of exogenous SP and C-fibre activation was proposed by Davis et al (19), who suggested that unmyelinated C-fibres constitute the afferent pathway of a reflex arc involving cholinergic neurons as the efferent pathway. Thus, release of both SP and acetylcholine evoked by C-fibre stimulation may accelerate the m.c. activity.

Antidromic trigeminal nerve stimulation has been shown to release SP (20). Electrical stimulation of the distal end of the isolated maxillary nerve causes an acceleration of m.c. activity, a response that is inhibited by the SP antagonist (D-Pro^{2}, D-Trp^{7,9})SP (21).

As far as we know, no morphological evidence has yet been presented for the existence of SP or SP-like peptides in the maxillary sinus in any species and all conclusions regarding the role of SP in the regulation of m.c. activity are based on the assumption that SP is present in the rabbit maxillary sinus and maxillary nerve to essentially the same extent as in the nose of other species (1, 2, 3). The aim of the present investigation was therefore to study, by immunocytochemistry, the occurrence and distribution of SP fibres in the mucosa of the maxillary sinus, in the maxillary nerve and in the sphenopalatine ganglion of the rabbit.

Materials and Methods

The sphenopalatine ganglia and specimens from the maxillary nerve and from the mucosa of the maxillary sinus were collected from 8 adult white rabbits of both sexes. The specimens were immersed in ice-cold buffered 4% formaldehyde solution overnight and rinsed in Tyrode solution containing 10% sucrose at 4 °C for 48 hours, frozen on dry ice, and sectioned (15 µ) in a cryostat at -20 °C.

Additional specimens from the maxillary nerve and sinus were fixed overnight in the same fixative as above. They were then immersed in a Tyrode solution containing 10% sucrose for 48 hours, briefly rinsed in a phosphate buffer and stretched on chrome-alum subbed microscope slides as whole mounts. Cryostat sections and whole mounts were processed for the immunocytochemical demonstration of SP using the indirect immunofluorescence method of Coons et al (22). A monoclonal SP antibody (code no. NC1 34) was obtained from Sera-lab, UK, and used in a dilution of 1:80 (cryostat sections) or in a dilution of 1:40 (whole mounts). The antibody has previously been used in immunocytochemical studies (23, 24). The site of the antigen-antibody reaction was revealed by fluorescein isothiocyanate-labelled goat anti-mouse IgG in a dilution of 1:20. Sections incubated with antibody inactivated by the addition of SP were used as controls.

Cross-reactivity with other structurally related peptides cannot be excluded. It is therefore appropriate to refer to the immunoreactive material as 'SP-like'. For brevity, however, we refer to the immunoreactive nerve fibres simply as SP fibres.

Results

A rich supply of SP containing nerve fibres was found in the subepithelial layer of the maxillary mucosa in all rabbits. The fibres formed a network (Fig. 1), and single varicose endings were sometimes found to penetrate into the epithelium. In the maxillary nerve numerous SP fibres were seen (Fig. 2).

The sphenopalatine ganglion was found as a thin leaf-like structure on the medial wall of the orbit, between the maxillary nerve and the bony wall. Fine beaded SP immunoreactive nerve fibres formed a network in the ganglion and frequently surrounded ganglion cells in a basket-like fashion. The ganglion cells themselves did not display any SP-like immunoreactivity (Fig. 3).

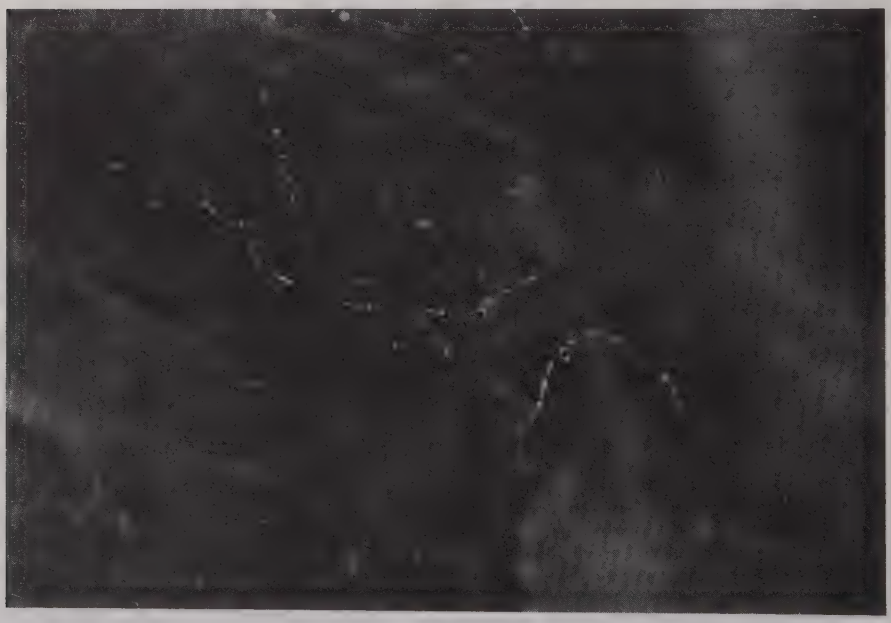

Fig. 1. Rabbit maxillary sinus. Whole mount preparation immunostained for SP. A network of fine beaded immunofluorescent nerve fibres is seen in the mucosa. (X 225).

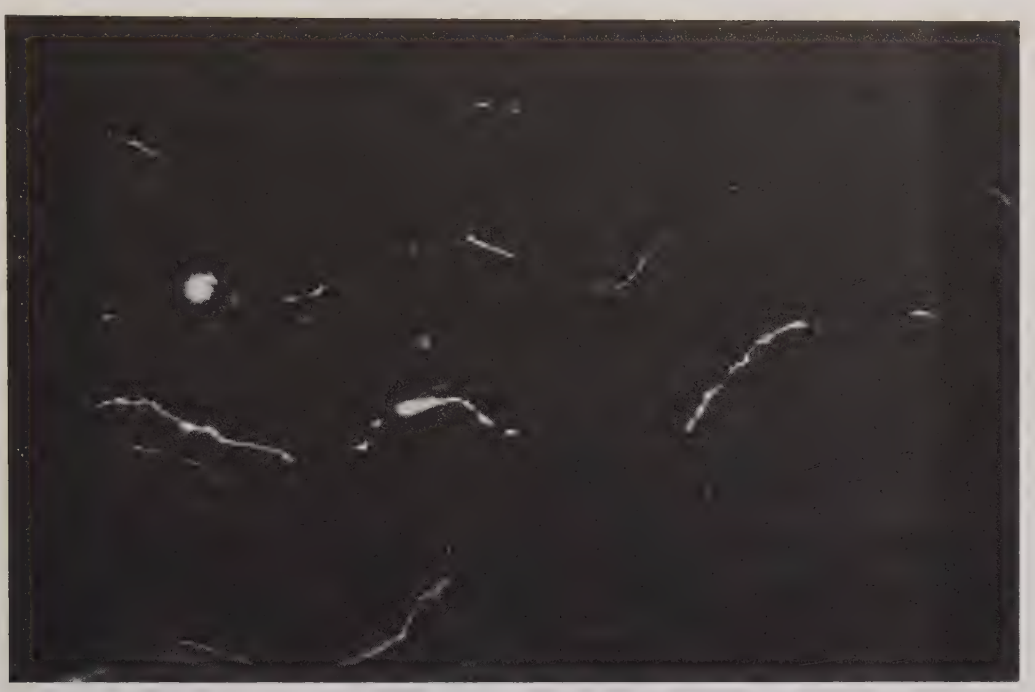

Fig. 2. Rabbit maxillary nerve. A dense supply of SP immunoreactive nerve fibres is seen. (X 225).

Fig. 3. The rabbit sphenopalatine ganglion. Numerous varicose SP fibres surround non-immunoreactive nerve cell bodies. (X 320).

Discussion

Studies regarding the occurrence of SP nerve fibres in the rabbit are sparse, mainly because most antisera against SP have been produced in that species with ensuing problems of intense unspecific binding of antibodies.

Monoclonal antibodies against SP made the present study possible, and we found that the occurrence of SP nerve fibres in the maxillary sinus was more abundant than expected from the previous results obtained in the rabbit nose (1). The monoclonal antibodies against SP used in the present study probably bind to the C-terminal sequence of the SP molecule (23). This sequence is common to other tachykinins, and it is possible that the SP antibody crossreacts with other peptides containing the same amino-acid sequence, such as SK. SP and SK have a common precursor peptide (25), and they probably coexist in the same neurons. Both SP- and SK-like immunoreactivity are released from guinea pig lung tissue after challenge with capsaicin (7). The effects of SK on m.c. activity seem to be similar to those of SP (Lindberg & Mercke, unpublished results).

Electron microscopical studies on the mucosa of the rabbit maxillary sinus (26) have revealed nerve terminals, mainly of unmyelinated type, to be in close contact with the epithelium. Intra-epithelial terminals containing small electron-lucent vesicles and also some large granular ones were seen, suggesting that the nerve endings contain both a "classical" (small molecule) transmitter and a neuropeptide. A similar finding with axons close to ciliated cells has been reported at all levels of extra-pulmonary airways in rat (27). In the light of the present finding, it is likely that some of these fibres contain SP.

The existence of SP nerve endings and nerve fibres in the rabbit sphenopalatine ganglion, which agrees with previous findings in other species (1, 3), gives a morphological basis for a local reflex arc involving both SP neurons and cholinergic effector neurons. Such a reflex arc could influence m.c. activity both through the release of SP and acetylcholine. However, it is quite possible that the reflex is mediated via the brainstem as well. On the other hand, a local regulation of autonomic ganglia influencing m.c. activity cannot be excluded. Such local regulation would parallel the postulated function of SP neurons in the ganglia of the enteric neuron system, see Pernow (28), where SP neurons are involved in the modulation of intestinal motility. Still another possibility for interactions between SP and cholinergic neurons is suggested by the finding that trigeminal branches themselves stain for acetylcholinesterase

activity (29). Thus acetylcholine may coexist with SP in primary sensory neurons.

C-fibres seem to be involved in the regulation of m.c. activity (15, 16, 21), vasodilation and plasma extravasation in the upper airways (10, 11). It has been reported that C-fibres in the lung are excited by various irritants and by inflammatory mediators like histamine and prostaglandins, see Widdicombe (30). It is not inconceivable that irritants or inflammatory mediators also accelerate m.c. activity via SP release. The results of the present investigation indicate the presence of SP in the rabbit maxillary sinus and favour the view that SP effects on m.c. activity are physiological and not merely pharmacological. The rabbit maxillary sinus thus emerges as an interesting experimental model for studying SP effects in the airway mucosa.

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REFLEX-INDUCED ACCELERATION OF MUCOCILIARY ACTIVITY AFTER SHORT-TERM EXPOSURE TO CIGARETTE SMOKE

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VII

Abstract

Substance P (SP), released from unmyelinated sensory C-fibres, is thought to accelerate mucociliary (m.c.) activity in the rabbit maxillary sinus. The accelerating effect of exogenous SP is resistant to atropine pretreatment. C-fibre endings are stimulated by various chemical irritants, including components of cigarette smoke. When delivered once a minute, 2.5 ml smoke puffs each accelerated the m.c. activity, the maximal increase being 34.7 $\pm$ 2.4% with a latency of 3.7 $\pm$ 0.5 sec.

The response to cigarette smoke was suppressed in rabbits pretreated with atropine, an SP antagonist ($D\text{-}Pro^{2}$, $D\text{-}Trp^{7,9}$)SP, capsaicin or hexamethonium. A response, albeit reduced, elicited in atropinized rabbits indicates a non-cholinergic component. The atropine-resistant acceleration was abolished by the SP antagonist. Together the results suggest that cigarette smoke accelerates m.c. activity through a reflex involving sensory SP containing C-fibres (afferent pathway) and cholinergic (parasympathetic) effector neurons (efferent pathway). Hence, the effect of cigarette smoke on the m.c. system reflects the joint release of both SP and acetylcholine. This dual mechanism may be of importance in the regulation of m.c. activity.

Introduction

The mucociliary (m.c.) activity is accelerated in vitro by several inflammatory mediators, e.g. prostaglandins, leukotriene-C_4 and histamine (1, 2). Inflammatory reaction involves a neurogenic component (3), probably mediated by the neuropeptide substance P (SP), which is released from the peripheral end of sensory C-fibres (4). M.c. activity in the rabbit maxillary sinus is accelerated by i.a. injection of SP (5), an effect which is resistant to atropine pretreatment. SP, or more appropriately SP-like immunoreactivity, is found in nerve fibres in the submucosa of the maxillary sinus and in fibres in the maxillary nerve of rabbits, as well as in the rabbit sphenopalatine ganglion (6). The presence of SP in nerve fibres in the upper airways has also been described in other species (7, 8). In the lower airways, SP-immunoreactive nerve fibres are numerous in the trachea and main bronchi (9, 10).

The SP-immunoreactive nerve fibres are sensory and of the unmyelinated C-fibre type (proposed by Hökfelt et al 1975) (11). However, it is possible that C-fibres may contain other peptides beside SP, including the related peptide substance K (SK) (12). Also, the coexistence of SP and a third peptide, calcitonin gene-related peptide (CGRP) has been demonstrated (13).

The effect of SP on the m.c. activity is mimicked by i.a. injection of bradykinin (14), a putative inflammatory mediator supposedly present in nasal secretions (15) and by i.a. injection of capsaicin (16). Both compounds are known to stimulate C-fibres in the lower airways (17, 18). In contrast to the effect of exogenous SP, atropine reduced the initial phase of the response to both bradykinin and capsaicin. Davis et al (19) have suggested that C-fibres constitute the afferent pathway of a secretory reflex with cholinergic nerve fibres as the efferent pathway. It is known that cholinergic agents increase m.c. activity in experimental animals (20, 21) and m.c. clearance in man (22). Stimulation of parasympathetic cholinergic neurons in the frog results in an immediate increase of m.c. activity and transport (21, 23). However, in our experiments atropine reduced only the rapid initial phase of the effect of bradykinin or capsaicin, while the entire response to the two drugs was abolished by pretreatment with the SP antagonist (D-Pro^2, D-Trp^{7,9})SP, known to inhibit the effect of exogenous SP on m.c. activity (14, 16, 24). These findings suggest that the response to bradykinin and capsaicin was induced by local release of both SP and acetylcholine. Furthermore, antidromic stimulation of sensory C-fibres accelerates m.c. activity (25). The effect of such stimulation was suppressed by atropine and abolished by the SP antagonist.

The activation of C-fibre endings has mainly been investigated in the lower airways. They are stimulated by endogenous mediators such as histamine, serotonin and prostaglandins and by inhaled irritants such as ammonia, volatile anesthetics like ether and some poison gases (see Widdicombe 1981) (26).

Cigarette smoke is a common inhaled irritant. Short-term exposure to cigarette smoke accelerates m.c. activity in the rabbit (27) and also m.c. clearance in humans (28, 29). The effect of cigarette smoke in the rabbit maxillary sinus was abolished by pretreatment with the ganglionic blocker hexamethonium and reduced by atropine, indicating that cigarette smoke either stimulates parasympathetic cholinergic neurons directly, or elicits a reflex with cholinergic nerve fibres as the efferent pathway (27).

Lundberg et al (30) found that cigarette smoke induced oedema in the rat nasal mucosa and produced a nose wiping behaviour in guinea pigs (31). Both responses were inhibited by pretreatment with high doses of capsaicin or an SP antagonist, suggesting that the responses were mediated via activation of SP neurons. Moreover, experiments on dogs have indicated that bronchial C-fibres are activated by cigarette smoke (32). Thus, the m.c. response to cigarette smoke may be a combined effect produced by SP released from the afferent pathway of the reflex arc (axon reflex), and acetylcholine from the cholinergic effector pathway.

The aim of the present study was to examine the effects of short-term exposure to cigarette smoke on m.c. activity. In order to analyze the parts played by SP and acetylcholine the following experimental design was followed:

1. Pretreatment with atropine (blockade of muscarinic receptors).

2. Pretreatment with the SP antagonist (D-Pro^{2}, D-Trp^{7,9})SP (blockade of SP receptors).

3. Pretreatment with capsaicin (desensitization of capsaicin--sensitive neurons).

4. Pretreatment with hexamethonium (ganglionic blockade).

Materials and Methods

The experiments were performed on 35 white rabbits of either sex weighing 2.1-2.7 kg. Details of anesthetic and surgical techniques have been published previously (33). The animals were anesthetized with urethane 2 g/kg i.m. as an initial dose, an extra dose of 0.5 g/kg was given i.v. during the operation. The arterial inlet for drugs was a retrograde cannula in the facial or lingual artery, continuously perfused with saline 2-5 ml/h. The mucosa in the rabbit maxillary sinus was exposed through a trepanation hole of about 2x8 mm, which was immediately covered with an antimist-window, and sealed with bone wax (Ethicon).

The m.c. activity (visible as flickering light reflections) was observed through a binocular microscope and the transportation of small particles like air bubbles, mucus clumps and shed cells was the criterion for a properly functioning preparation. One of the eyepieces was then switched to a phototransducer and the m.c. activity recorded by a photoelectric technique (34). The m.c. wave pattern was monitored continuously on an oscilloscope and recorded on an ink writer. The recordings were analyzed by a computerized frequency calculator, which expressed the m.c. beat frequency in waves/min and calculated every 10 sec during the experiments and at intervals of 1-5 min otherwise. The induced frequency changes were expressed as percentages of the basal m.c. wave frequency (frequency zero level) immediately preceding the administration of cigarette smoke. ECG and rectal temperature were monitored and body temperature maintained at 37.0-38.5 ^{o}C by a heating pad.

Cigarette smoke was administered according to Hybbinette (27). Smoke was drawn by a 20 ml syringe from a commercial cigarette (Pall Mall, a king-size cigarette without filter) and was ejected through a polyethylene catheter (inner diameter 1.5 mm) with its tip 2-3 mm in front of the nostril on the same side as the trepanized sinus unless otherwise stated. The cigarettes were smoked to a butt length of approximately 25 mm. According to the manufacturer the smoke from 1 cigarette contains about 1.5 mg nicotine, 21 mg tar and 13 mg carbon monoxide.

The smoke was administered during 2-4 consecutive inhalations once a minute as a 2.5 ml puff from the syringe (unless otherwise stated). It was easy to discern whether the rabbit had in fact inhaled cigarette smoke by observing respiratory reflexes (reduction in breathing or even apnoea) (35, 36).

The following drugs were tested: Atropine (ACO, Sweden), (D-Pro^2, D-Trp^{7,9})SP (Ferring, Sweden), capsaicin, hexamethonium and methacholine (Sigma, USA). Capsaicin was dissolved in saline containing 10% Tween 80 and 10% ethanol, further dilutions were made in saline. The other drugs were dissolved in saline, except (D-Pro^2, D-Trp^{7,9})SP which was dissolved in saline containing 0.5% albumine (Kabi, Sweden) in order to prevent adsorption to glass and plastic surfaces.

Experimental procedure. The m.c. activity was recorded once a minute until the frequency had stabilized (usually for a period of 30 min) before the experiments with cigarette smoke were commenced. The effect of cigarette smoke without any pretreatment was first determined in all animals. Experiments with room air delivered through the syringe served as controls (these controls were not run in all animals). The animals breathed room air between experiments.

In preliminary experiments the effect of four different pretreatments (0.2 mg/kg atropine, 1 mg/kg (D-Pro^2, D-Trp^{7,9})SP, 2 mg/kg hexamethonium or repeated injections of capsaicin) were compared in 2-3 rabbits each. Since it was found that the effect of the antagonists declined with time, the studies were limited to the effect of 3 consecutive puffs. Cigarettes were first smoked to 40 mm butt length in a fume cupboard and the smoke produced from 40 to 25 mm butt length (sufficient for 3-4 puffs) was then used for the following experimental series:

1. A simple dose-response curve was obtained by diluting cigarette smoke with room air. The effect of undiluted smoke and of dilutions of 1:4 and 1:1 were compared.

2. The effect of cigarette smoke applied to the contralateral nostril was recorded and compared with that of ipsilaterally applied smoke (by switching the polyethylene catheter tip to the contralateral side).

3. Rabbits were pretreated with 0.2 mg/kg atropine (volume 0.2 ml) i.a. 2 min prior to challenge with cigarette smoke. 0.5 µg/kg methacholine (volume 0.2 ml) was injected 5-10 min after the last challenge in the series.

4. The SP antagonist (D-Pro^2, D-Trp^{7,9})SP was given as an arterial infusion 1 mg/kg (1 mg/ml) during 4 min. Smoke was then delivered beginning 10 min after interruption of the infusion.

5. Capsaicin was given repeatedly i.a. once every minute according to the following dose schedule in order to desensitize capsaicin-sensitive C-fibres: 200 µgx5, 600 µgx5 and 1.8 mgx5 (volumes 0.2 ml) (recommended by J. Szolcsányi, personal communication). The time interval between application of the different doses was 10 min. Cigarette smoke was administered 10 min after the last injection of 1.8 mg capsaicin.

6. Hexamethonium 2 mg/kg (volume 0.2 ml) was given i.a. 1 min before exposure to smoke.

7. In a final series of experiments the effect of pretreatment with atropine alone or atropine + (D-Pro^2, D-Trp^{7,9})SP was compared in the following way: Animals were pretreated with 2 mg/kg atropine i.v. and 0.2 mg/kg i.a. at 10 min and 2 min respectively before exposure to smoke with an interval of 2 min between smoke challenges (with in these cases 5 consecutive smoke puffs from a whole cigarette). The interval of 2 min was chosen in order to make it possible for the m.c. wave frequency to return to baseline between challenges. Methacholine 0.5 µg/kg (volume 0.2 ml) was given 5 min after the last smoke puff in the series. One hour later atropinization was repeated together with i.a. infusion of (D-Pro^2, D-Trp^{7,9})SP 1 mg/kg (1 mg/ml) for 4 min before further exposure to smoke. Again 0.5 µg/kg methacholine was injected 5 min after the last smoke puff. Control experiments were carried out in 3 rabbits that received a saline infusion (containing 0.5% albumin) instead of the SP antagonist.

The results were expressed as means $\pm$ standard error of the mean (SEM), unless otherwise stated. The statistical treatment was two-way analysis of variance, except when the effects of the different antagonists were compared (one-way analysis of variance). P-values <0.05 were considered significant.

Results

Exposure to cigarette smoke increased the m.c. wave frequency dose-dependently (see Fig. 1), the maximum increase being 34.7 ± 2.4% (n=25) for undiluted smoke. The corresponding value for the controls receiving room air was 4.0 ± 0.9% (n=13). The increase was seen almost immediately after exposure, the latency of the response being 3.7 ± 0.5 sec (n=5). The latency was calculated from the ink writer recordings and defined as the time interval between challenge and a frequency increase of more than 10%. Maximal frequency increases were noticed within 20 sec after exposure in untreated animals. Results were reproducible in the same animal, although in most rabbits the response to the very first smoke puff was weaker than the subsequent ones.

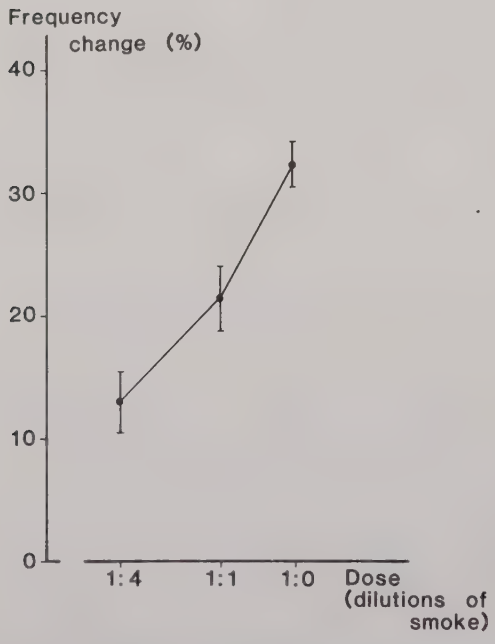

Fig. 1. The effect of tobacco smoke on m.c. activity. Two different dilutions in room air are compared with undiluted smoke. The results are expressed as means and SEM (standard error of mean). The correlation coefficient for raw data: r=0.80 (n=5). Frequency zero level was 1383 ± 124 waves/min (mean ± SD).

M.c. activity was accelerated also by contralateral challenge with smoke, the frequency increase being 19.0 ± 3.5% compared to 28.3 ± 3.9% after ipsilateral challenge, p=0.06 (n=5). Furthermore, in two other rabbits, which after several exposures to smoke ceased to respond because of oedema in the nasal mucosa impairing nasal patency, it was still possible to evoke the expected increase by switching the tip of the catheter to the contralateral side.

Neither atropine, hexamethonium nor (D-Pro^2, D-Trp^{7,9})SP had any effect *per se* on the m.c. activity. Capsaicin stimulated the m.c. activity strongly and there was no apparent tachyphylaxis to the drug (Fig. 2). The peak increases were 30.3 ± 4.3% (for the dose 200 µg), 44.2 ± 5.9% (600 µg) and 48.1 ± 11.0% (1.8 mg), $p<0.001$ (n=6). However, the responses were of short duration and the influence on the frequency after 10 min (when the experiments with smoke commenced) was not significant: from 1306 ± 151 (mean ± SD) before capsaicin treatment to 1380 ± 223 (mean ± SD) at the first smoke challenge, $p=0.16$ (n=6).

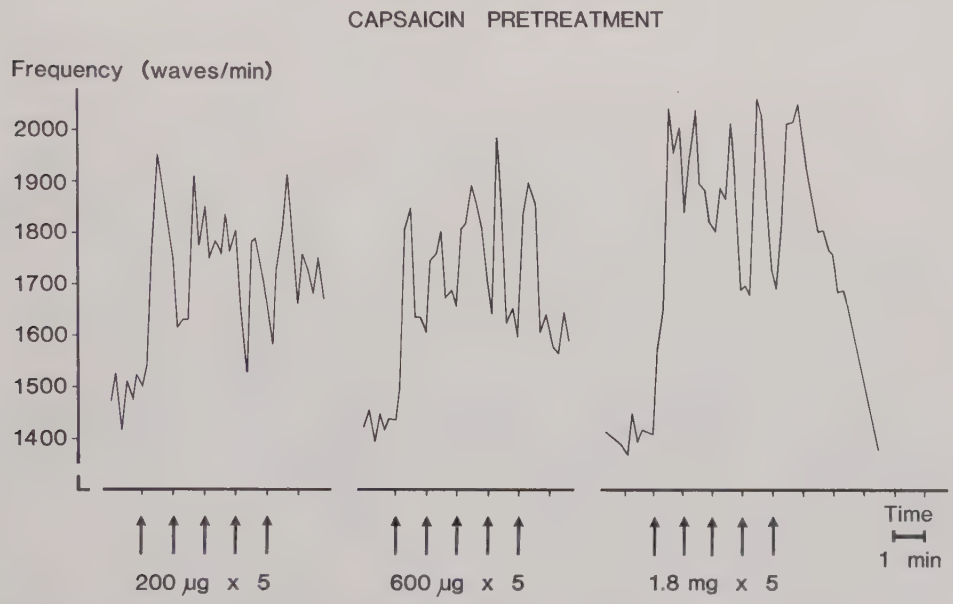

Fig. 2. An example of the effect of capsaicin pretreatment *per se* in one rabbit given in order to desensitize capsaicin-sensitive C-fibres. Each arrow represents the i.a. injection of a 0.2 ml bolus dose of capsaicin.

All three antagonists and also capsaicin pretreatment suppressed the subsequent response to cigarette smoke (Figs. 3-6). However, after pretreatment with atropine or (D-Pro^2, D-Trp^{7,9})SP, the duration of the suppression was short and the effect of these drugs declined with each exposure to smoke (cf. time-course of the response in Figs. 3-6, right side). Atropine was significantly less effective than (D-Pro^2, D-Trp^{7,9})SP, $p<0.05$ and hexamethonium, $p<0.01$ (one-way analysis of variance). There were no other statistically significant differences between the effects of the various pretreatments.

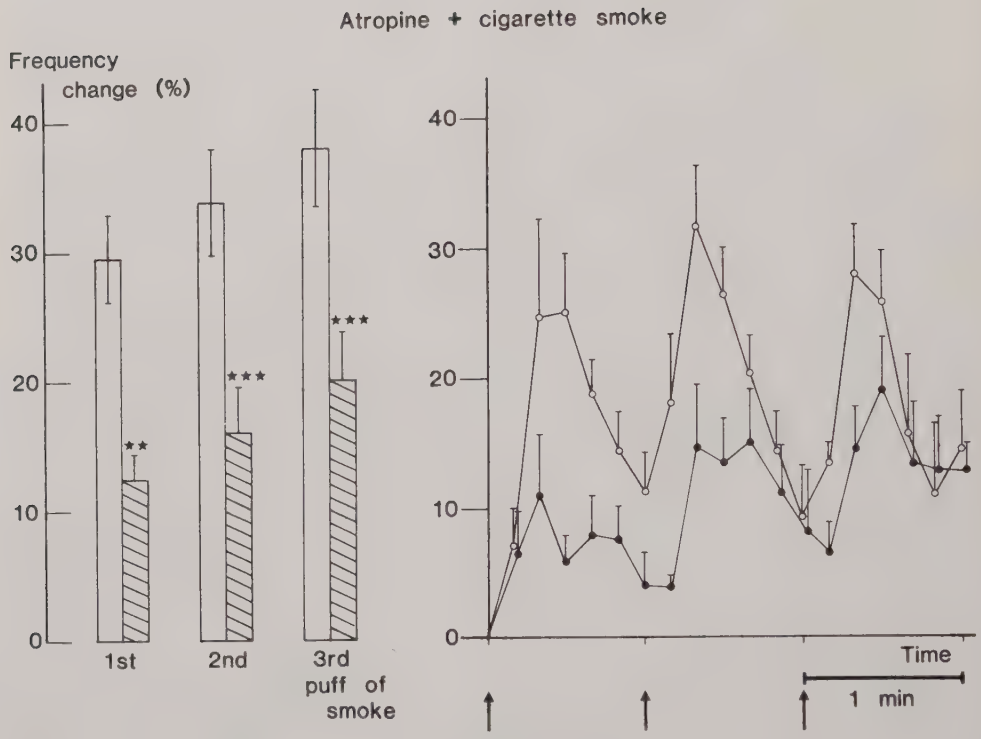

Fig. 3. The effect of pretreatment with 0.2 mg/kg atropine on the m.c. response to three challenges with tobacco smoke at intervals of one min. Left: ⌐¬ without pretreatment, ◹ after pretreatment (n=11). Right: time-course without (○——○) and after (●——●) pretreatment in the seven rabbits that did not respond to challenges with methacholine 5 min after the latest smoke puff (arrow = smoke challenge). The results are expressed as means and SEM. Frequency zero level was 1283 ± 220 waves/min (mean ± SD). ★★ = $p<0.01$, ★★★ = $p<0.001$.

It was found that 4 of 11 rabbits pretreated with 0.2 mg/kg atropine showed a weak response to methacholine given 5 min after the latest challenge with smoke, suggesting that the m.c. response in these rabbits was due to a gradually diminishing muscarinic blockade, rather than to a non-cholinergic mechanism. However, rabbits which were adequately atropinized, as evidenced by the lack of response to methacholine, still responded to cigarette smoke with an accelerated m.c. activity, albeit reduced compared with control experiments (Fig. 3 right side, Fig. 7).

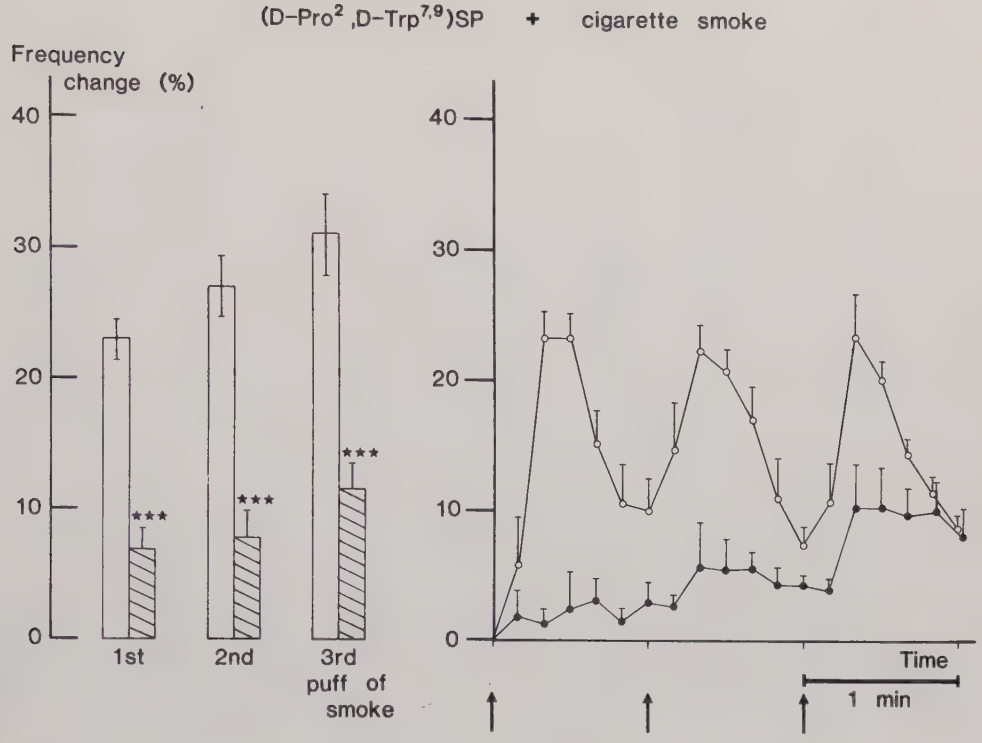

Fig. 4. The effect of pretreatment with 1.0 mg/kg (D-Pro^2, D-Trp^{7,9})SP on the m.c. response to three challenges with tobacco smoke at intervals of one min. Left: ⌜¬⌝ without pretreatment, ⌜\⌝ after pretreatment (n=8). Right: time-course without (○——○) and after (●——●) pretreatment (n=7) (arrow = smoke challenge). The results are expressed as means and SEM. Frequency zero level was 1377 ± 126 waves/min (mean ± SD).
★★★ = $p<0.001$.

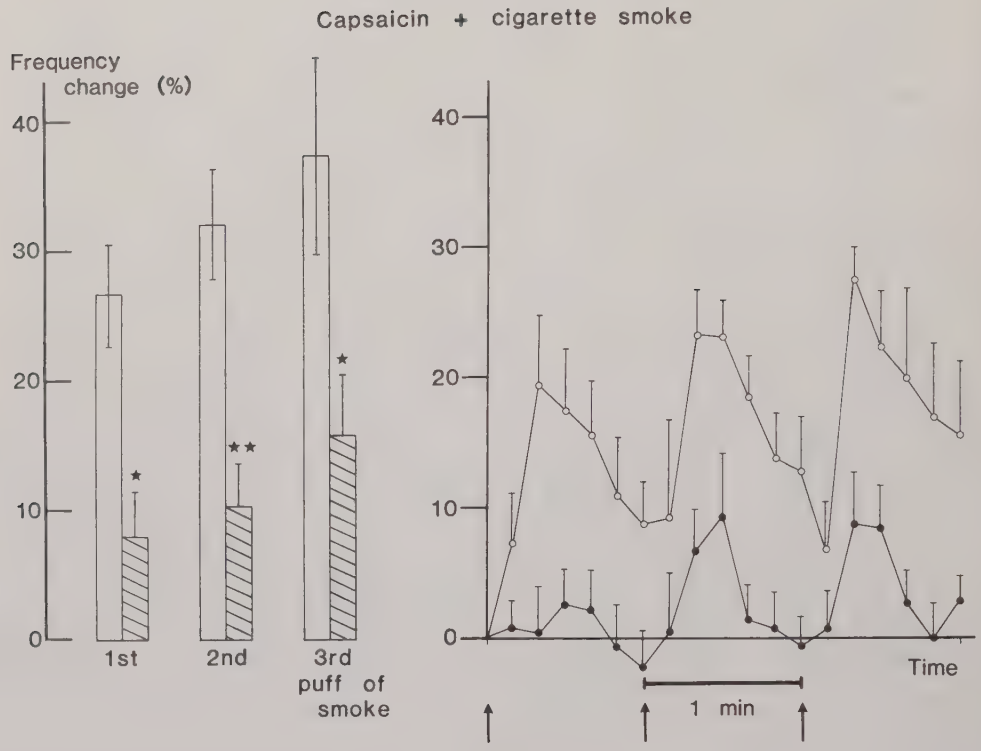

Fig. 5. The effect of pretreatment with increasing doses of capsaicin on the m.c. response to three challenges with tobacco smoke at intervals of one min. Left: ⌐¬ without pretreatment, ⌐◥ after pretreatment (n=6). Right: time-course without (○——○) and after (●——●) pretreatment (n=5) (arrow = smoke challenge). The results are expressed as means and SEM. Frequency zero level was 1316 ± 216 waves/min (mean ± SD).
★ = $p<0.05$, ★★ = $p<0.01$.

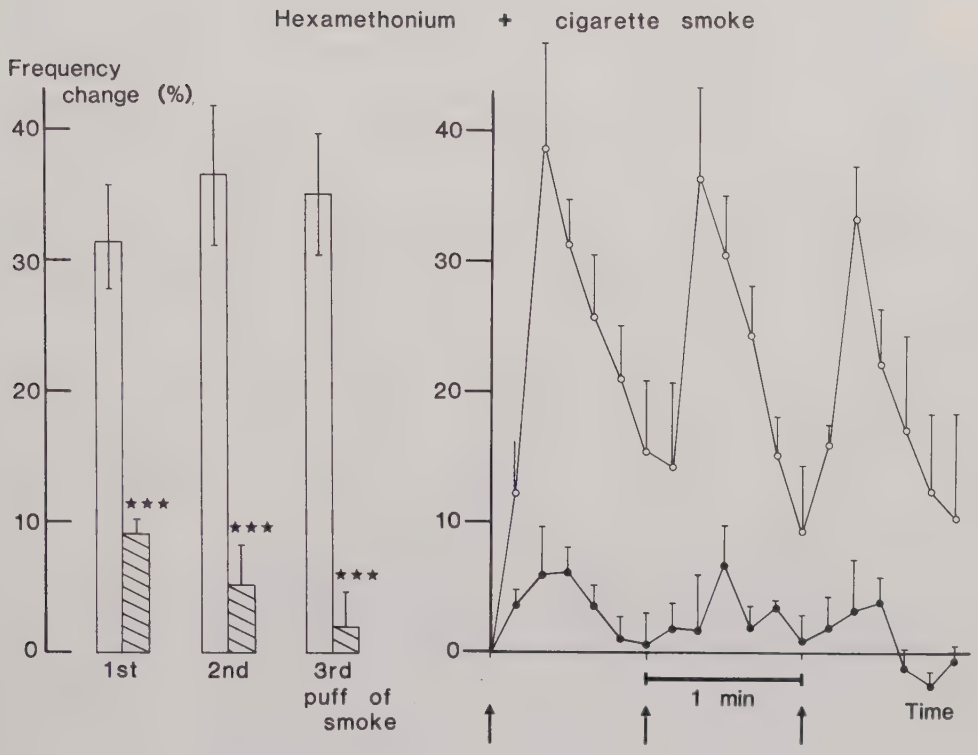

Fig. 6. The effect of pretreatment with 2.0 mg/kg hexamethonium on the m.c. response to three challenges with tobacco smoke at intervals of one min. Left: ⌐¬ without pretreatment, ◹ after pretreatment (n=8). Right: time-course without (○——○) and after (●——●) pretreatment (n=5) (arrow = smoke challenge). The results are expressed as means and SEM. Frequency zero level was 1154 ± 159 waves/min (mean ± SD).
★★★ = $p<0.001$.

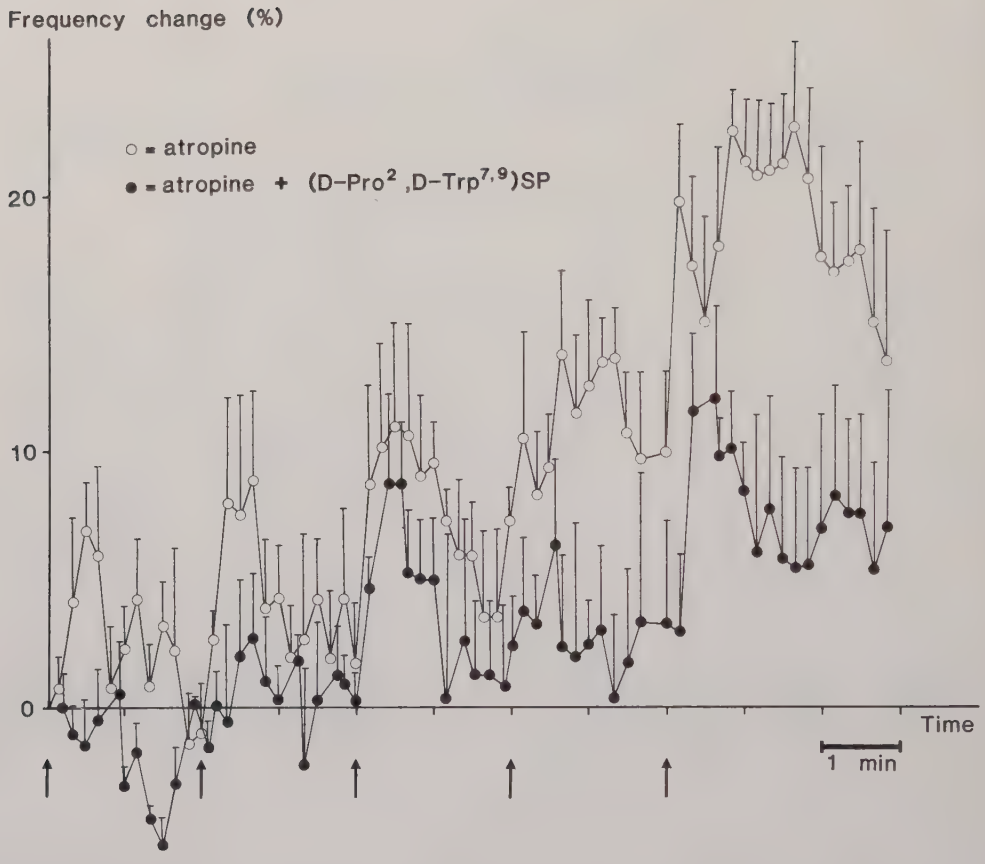

Fig. 7. Despite the combined i.v. (2.0 mg/kg) and i.a. (0.2 mg/kg) pretreatment with atropine (○———○) smoke accelerates m.c. activity (each arrow represents one smoke puff). The combined pretreatment with both atropine and 1.0 mg/kg (D-Pro^2, D-Trp^{7,9})SP (●———●) inhibits the non-cholinergic response, $p<0.001$ (n=5). The results are expressed as means and SEM. Frequency zero levels were 1210 ± 216 waves/min (atropine only) and 1221 ± 100 waves/min (atropine + (D-Pro^2, D-Trp^{7,9})SP).

Combined i.v. and i.a. atropine pretreatment confirmed the existence of an atropine-resistant m.c. response to smoke. Methacholine injected afterwards had no effect in these rabbits. Repeated atropinizations in these rabbits gave the same type of response to smoke. If (D-Pro^2, D-Trp^{7,9})SP was added to the muscarinic blockade in these rabbits, a more prolonged and stronger inhibition of the response to smoke was seen, $p<0.001$ ($n=5$) (Fig. 7). However, also this combined blockade gradually diminished with time, as evidenced by the returning responsiveness to cigarette smoke.

The three control rabbits that received a saline infusion instead of (D-Pro^2, D-Trp^{7,9})SP, responded to cigarette smoke with the anticipated increase of m.c. activity. The maximum increase was 29.6 $\pm$ 7.5% after atropinization only, and 39.5 $\pm$ 13.7% after atropine and saline in combination.

Discussion

Cigarette smoke given in volumes and at time intervals corresponding to cigarette smoking in man (37) accelerated m.c. activity in the rabbit maxillary sinus. Increased m.c. clearance after short-term exposure to cigarette smoke has previously been reported (28, 29). In the present investigation the m.c. effect can probably be explained by a nervous reflex influencing the m.c. system, since it was also evoked by smoke applied to the contralateral nostril.

The effect of cigarette smoke was inhibited by pretreatment with the SP antagonist ($D\text{-}Pro^{2}$, $D\text{-}Trp^{7,9}$)SP (Fig. 4) and by desensitization of capsaicin-sensitive neurons after repeated administration of capsaicin (Fig. 5) suggesting that the response involved C-fibre stimulation. Although pretreatment with atropine clearly reduced the response (Fig. 3), indicating that part of the response was mediated through cholinergic effector neurons, a response to cigarette smoke remained in atropinized animals. The non-cholinergic response could be inhibited by the addition of the SP antagonist (Fig. 7) supporting the view that exposure to cigarette smoke activates a reflex arc involving C-fibres (containing SP and/or SP-like peptides) as the afferent and cholinergic effector neurons as the efferent pathway. Taken together with the strong accelerating effect of SP on m.c. activity (5), these results indicate that the m.c. response to cigarette smoke reflects the local release of both SP and acetylcholine.

The ganglionic blocker hexamethonium abolished the m.c. response to cigarette smoke, a finding first described by Hybbinette (27). This seems to disagree with the hypothesis that part of the response is mediated by SP, as the effect of exogenous SP is not blocked by hexamethonium (16). However, C-fibre activation by nicotine applied to the nasal mucosa is inhibited by hexamethonium (38, 39), as well as effects in the nasal mucosa of cigarette smoke (30). It has been suggested that hexamethonium--sensitive nicotinic receptors may exist on peripheral branches of sensory nerves as well as in autonomic ganglia influencing airway responses to cigarette smoke (30). In this context it is interesting that intraarterial nicotine produces a transient very rapid acceleration of m.c. activity, which is inhibited by atropine and hexamethonium (40). C-fibres may also be stimulated by several of the 4000 other components of cigarette smoke (41) besides nicotine, although a nicotine-free herb cigarette did not induce m.c. acceleration (27). Cigarette smoke contains ammonia and various aldehydes which stimulate C-fibres in the lower airways. Identification of the components in cigarette smoke which are responsible for the m.c. acceleration requires

further investigation.

The effects of SP upon release from unmyelinated C-fibres on the m.c. system is not known in detail. It is conceivable that SP alters the composition of the mucus, since *in vitro* experiments using secretory cells from the canine airways have shown SP to stimulate mucin production (42). The rapid effect on m.c. activity could be explained by a change in the periciliary layer. This is supported by the fact that SP has been found to stimulate net chloride secretion from the canine trachea and increase short circuit current within 3-5 sec of administration despite pre-treatment of tissues with atropine or adrenergic antagonists (43). In contrast it has been reported that cigarette smoke decreases short circuit current in canine tracheal epithelium both *in vivo* and *in vitro* (44), in turn suggesting impaired m.c. activity. This discrepancy may be due to the fact that the gas exchange between the nose and the maxillary sinus is slow (45) and it is therefore unlikely that the epithelium in the rabbit maxillary sinus is itself in immediate contact with the smoke. If this assumption is correct, the non-cholinergic part of the increased m.c. activity induced by cigarette smoke through release of SP emerges as an axon reflex in the classical sense.

Effects of cigarette smoke on the nasal mucosa of the rabbit were investigated by Pell et al (46). Smoke induced release of glycoproteins, which further suggests that the stimulation of m.c. activity after short-term exposure to cigarette smoke could be explained by a change in the composition of the airway secretions, possibly in the periciliary layer, in turn causing a more vigorous ciliary beating.

The findings of the present investigation indicate that short--term exposure to cigarette smoke accelerates m.c. activity through activation of a protective reflex involving the local release of SP (and/or SP-like peptides) from the peripheral endings of the C-fibres (axon reflex) and local release of acetylcholine from parasympathetic nerve fibres. C-fibre receptors in the airways are stimulated by irritants and endogenous mediators (see Widdicombe 1981 and Coleridge & Coleridge 1984) (26, 47). In other words, C-fibre endings seem to be activated by any noxious stimuli. Increased m.c. activity under these conditions could contribute substantially to the defence of the respiratory tract.

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